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TITLE OF THE INVENTION (280 characters max)

GENE REGULATION IN TRANSGENIC ANIMALS USING A TRANSPOSON-BASED VECTOR

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Respectfully submitted,

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GENE REGULATION IN TRANSGENIC ANIMALS USING A TRANSPOSON-BASED VECTOR

The U.S. Government has certain rights in this invention. The development of this invention was partially funded by the United States Government under a HATCH grant from the United States Department of Agriculture, partially funded by the United States Government with Formula 1433 funds from the United States Department of Agriculture and partially funded by the United States Government under contract DAAD 19-02016 awarded by the Army.

FIELD OF THE INVENTION

The present invention relates generally to cell-specific gene regulation in transgenic animals. Animals may be made transgenic through administration of a transposon-based vector through any method of administration including pronuclear injection, or intraembryonic, intratesticular, intraoviductal, intraovarian or intravenous administration. In some embodiments, the transposon-based vector is administered to the reproductive tract in an animal. The reproductive tract includes an ovary, ova within an ovary, and an oviduct. Such administration results in incorporation of a gene of interest contained in the vector in the ovary, the oviduct or an ovum of the animal. These transgenic animals contain the gene of interest in all cells, including germ cells. Animals may also be made transgenic by targeting specific cells for uptake and gene incorporation of the transposon-based vectors. Stable incorporation of a gene of interest into cells of the transgenic animals is demonstrated by expression of the gene of interest in a cell, wherein expression is regulated by a promoter sequence. The promoter sequence may be provided as a transgene along with the gene of interest or may be endogenous to the cell. The promoter sequence may be

constitutive or inducible, wherein inducible promoters include tissue-specific promoters, developmentally regulated promoters and chemically inducible promoters.

BACKGROUND OF THE INVENTION

Transgenic animals are desirable for a variety of reasons, including their potential as biological factories to produce desired molecules for pharmaceutical, diagnostic and industrial uses. This potential is attractive to the industry due to the inadequate capacity in facilities used for recombinant production of desired molecules and the increasing demand by the pharmaceutical industry for use of these facilities. Numerous attempts to produce transgenic animals have met several problems, including low rates of gene incorporation and unstable gene incorporation. Accordingly, improved gene technologies are needed for the development of transgenic animals for the production of desired molecules.

Improved gene delivery technologies are also needed for the treatment of disease in animals and humans. Many diseases and conditions can be treated with gene-delivery technologies, which provide a gene of interest to a patient suffering from the disease or the condition. An example of such disease is Type 1 diabetes. Type 1 diabetes is an autoimmune disease that ultimately results in destruction of the insulin producing β-cells in thc pancreas. Although patients with Type 1 diabetes may be treated adequately with insulin injections or insulin pumps, these therapies are only partially effective. Insulin replacement, such as via insulin injection or pump administration, cannot fully reverse the defect in the vascular endothelium found in the hyperglycemic state (Pieper et al., 1996. Diabetes Res. Clin. Pract. Suppl. S157-S162). In addition, hyper- and hypoglycemia occurs frequently despite intensive home blood glucose monitoring. Finally, careful dietary constraints are needed to maintain an adequate ratio of calories consumed. This often causes major psychosocial stress for many diabetic patients. Development of gene therapies providing delivery of the insulin gene into the pancreas of diabetic patients could overcome many of these problems and result in improved life expectancy and quality of life.

Several of the prior art gene delivery technologies employed viruses that are associated with potentially undesirable side effects and safety concerns. The majority of current gene-delivery technologies useful for gene therapy rely on virus-based delivery vectors, such as adeno and adeno-associated viruses, retroviruses, and other viruses, which have been attenuated to no longer replicate. (Kay, M.A., et al. 2001. Nature Medicine 7:33-40).

There are multiple problems associated with the use of viral vectors. Firstly, they are not tissue-specific. In fact, a gene therapy trial using adenovirus was recently

halted because the vector was present in the patient's sperm (Gene trial to proceed despite fears that therapy could change child's genetic makeup. The New York Times, December 23, 2001). Secondly, viral vectors are likely to be transiently incorporated, which necessitates re-treating a patient at specified time intervals. (Kay, M.A., et al. 2001. Nature Medicine 7:33-40). Thirdly, there is a concern that a viral-based vector could revert to its virulent form and cause disease. Fourthly, viral-based vectors require a dividing cell for stable integration. Fifthly, viral-based vectors indiscriminately integrate into various cells, which can result in undesirable germline integration. Sixthly, the required high titers needed to achieve the desired effect have resulted in the death of one patient and they are believed to be responsible for induction of cancer in a separate study. (Science, News of the Week, October 4, 2002).

Accordingly, what is needed is a new meothd to produce transgenic animals and humans with stably incorporated genes, in which the vector containing those genes does not cause disease or other unwanted side effects. There is also a need for DNA constructs that would be stably incorporated into the tissues and cells of animals and humans, including cells in the resting state, that are not replicating. There is a further recognized need in the art for DNA constructs capable of delivering genes to specific tissues and cells of animals and humans.

When incorporating a gene of interest into an animal for the production of a desired protein or when incorporating a gene of interest in an animal or human for the treatment of a disease, it is often desirable to selectively activate incorporated genes using inducible promoters. These inducible promoters are regulated by substances either produced or recognized by the transcription control elements within the cell in which the gene is incorporated. In many instances, control of gene expression is desired in transgenic animals or humans so that incorporated genes are selectively activated at desired times and/or under the influence of specific substances. Accordingly, what is needed is a means to selectively activate genes introduced into the genome of cells of a transgenic animal or human. This can be taken a step further to cause incorporation to be tissue-specific, which prevents widespread gene incorporation throughout a patient's body (animal or human). This decreases the amount of DNA needed for a treatment, decreases the chance of incorporation in gametes, and targets gene delivery, incorporation, and expression to the desired tissue where the gene is needed to function. What is also needed is a rapid expression method for rapidly producing a protein or peptide of interest in eggs and milk of transgenic animals.

SUMMARY OF THE INVENTION

The present invention addresses the problems described above by providing new, effective and efficient compositions for producing transgenic animals and for treating disease in animals or humans. Transgenic animals include all egg-laying animals and milk-producing animals. Transgenic animals further include but are not limited to avians, fish, amphibians, reptiles, insects, mammals and humans. In another preferred embodiment, the animal is a milk-producing animal, including but not limited to bovine, porcine, ovine and equine animals. In a preferred embodiment, the animal is an avian animal. In another preferred embodiment, the animal is a mammal. Animals are made transgenic through administration of a composition comprising a transposon-based vector designed for incorporation of a gene of interest for production of a desired protein, together with an acceptable carrier. A transfection reagent is optionally added to the composition before administration.

The transposon-based vectors of the present invention include a transposase, operably-linked to a first promoter, and a coding sequence for a protein or peptide of interest operably-linked to a second promoter, wherein the coding sequence for the protein or peptide of interest and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based vector also includes the following characteristics: a) one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:1) at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the transposase, wherein the nucleotide at the third base position of each codon is changed to an A or a T without changing the corresponding amino acid; c) addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene. In some embodiments, the effective polyA sequence is an avian optimized polyA sequence.

Use of the compositions of the present invention results in highly efficient and stable incorporation of a gene of interest into the genome of transfected animals. For example, transgenic avians have been mated and produce transgenic progeny in the G1 generation. The transgenic progeny have been mated and produce transgenic progeny in the G2 generation.

The present invention also provides for tissue-specific incorporation and/or expression of a gene of interest. Tissue-specific incorporation of a gene of interest may be achieved by placing the transposase gene under the control of a tissue-specific promoter, whereas tissue-specific expression of a gene of interest may be achieved by placing the gene of interest under the control of a tissue-specific promoter. In some embodiments, the gene of interest is transcribed under the influence of an ovalbumin, or other oviduct specific, promoter. Linking the gene of interest to an oviduct specific promoter in an egg-laying animal results in synthesis of a desired molecule and deposition of the desired molecule in a developing egg.

In some embodiments, compositions of the present invention are introduced into the reproductive system of an animal. The compositions of the present invention are administered to a reproductive organ including, but not limited to, an oviduct, an ovary, or into the duct system of the mammary gland. The compositions of the present invention are may be administered to a reproductive organ of an animal through the cloaca. The compositions of the present invention may be directly administered to a reproductive organ or can be administered to an artery leading to the reproductive organ. In a preferred embodiment, the compositions of the present invention are introduced into the the reproductive system of an avian animal. In another preferred embodiment, the compositions of the present invention are introduced into the the intramammary duct system of a mammal. Transcription of the gene of interest in the epithelial cells of the mammary gland results in synthesis of a desired molecule and deposition of the desired molecule in the milk. A preferred molecule is a protein. In some embodiments, the desired molecule deposited in the milk is an antiviral protein, an antibody, or a serum protein.

In other embodiments, specific incorporation of the proinsulin gene into liver cells of a diabetic animal results in the improvement of the animal's condition. Such improvement is achieved by placing a transposase gene under the control of a liver-specific promoter, which drives integration of the gene of interest in liver cells of the diabetic animal.

The present invention advantageously produces a high number of transgenic animals having a gene of interest stably incorporated. These transgenic animals successfully pass the desired gene to their progeny. The transgenic animals of the present invention also produce large amounts of a desired molecule encoded by the

transgene. Transgenic egg-laying animals, particularly avians, produce large amounts of a desired protein that is deposited in the egg for rapid harvest and purification. Transgenic milk-producing animals produce large amounts of a desired protein that is deposited in the milk for rapid harvest and purification.

Any desired gene may be incorporated into the novel transposon-based vectors of the present invention in order to synthesize a desired molecule in the transgenic animals. Proteins, peptides and nucleic acids are preferred desired molecules to be produced by the transgenic animals of the present invention. Particularly preferred proteins are antibody proteins and other immunopharmecuetical proteins.

This invention provides a composition useful for the production of transgenic hens capable of producing substantially high amounts of a desired protein or peptide. Entire flocks of transgenic birds may be developed very quickly in order to produce industrial amounts of desired molecules. The present invention solves the problems inherent in the inadequate capacity of fermentation facilities used for bacterial production of molecules and provides a more efficient and economical way to produce desired molecules. Accordingly, the present invention provides a means to produce large amounts of therapeutic, diagnostic and reagent molecules.

Transgenic chickens are excellent in terms of convenience and efficiency of manufacturing molecules such as proteins and peptides. Starting with a single transgenic rooster, thousands of transgenic offspring can be produced within a year. (In principle, up to forty million offspring could be produced in just three generations). Each transgenic female is expected to lay at least 250 eggs/year, each potentially containing hundreds of milligrams of the selected protein. Flocks of chickens numbering in the hundreds of thousands are readily handled through established commercial systems. The technologies for obtaining eggs and fractionating them are also well known and widely accepted. Thus, for each therapeutic, diagnostic, or other protein of interest, large amounts of a substantially pure material can be produced at relatively low incremental cost.

A wide range of recombinant peptides and proteins can be produced in transgenic egg-laying animals and milk-producing animals. Enzymes, hormones, antibodies, growth factors, serum proteins, commodity proteins, biological response modifiers, peptides and designed proteins may all be made through practice of the present invention. For example, rough estimates suggest that it is possible to produce

in bulk growth hormone, insulin, or Factor VIII, and deposit them in egg whites, for an incremental cost in the order of one dollar per gram. At such prices it is feasible to consider administering such medical agents by inhalation or even orally, instead of through injection. Even if bioavailability rates through these avenues were low, the cost of a much higher effective-dose would not be prohibitive.

In one embodiment, the egg-laying transgenic animal is an avian. The method of the present invention may be used in avians including Ratites, Psittaciformes, Falconiformes, Piciformes, Strigiformes, Passeriformes, Coraciformes, Ralliformes, Cuculiformes, Columbiformes, Galliformes, Anseriformes, and Herodiones. Preferably, the egg-laying transgenic animal is a poultry bird. More preferably, the bird is a chicken, turkey, duck, goose or quail. Another preferred bird is a ratite, such as, an emu, an ostrich, a rhea, or a cassowary. Other preferred birds are partridge, pheasant, kiwi, parrot, parakeet, macaw, falcon, eagle, hawk, pigeon, cockatoo, song birds, jay bird, blackbird, finch, warbler, canary, toucan, mynah, or sparrow.

In another embodiment, the transgenic animal is a milk-producing animal, including but not limited to bovine, ovine, porcine, equine, and primate animals. Milk-producing animals include but are not limited to cows, goats, horses, pigs, buffalo, rabbits, non-human primates, and humans.

Accordingly, it is an object of the present invention to provide novel transposon-based vectors.

It is another object of the present invention to provide novel transposon-based vectors that encode for the production of desired proteins or peptides in cells.

It is an object of the present invention to produce transgenic animals through administration of a transposon-based vector.

Another object of the present invention is to produce transgenic animals through administration of a transposon-based vector, wherein the transgenic animals produce desired proteins or peptides.

Yet another object of the present invention is to produce transgenic animals through administration of a transposon-based vector, wherein the transgenic animals produce desired proteins or peptides and deposit the proteins or peptides in eggs or milk.

It is a further object of the present invention to produce transgenic animals through intraembryonic, intratesticular, intraovarian, intravenous or intraoviductal administration of a transposon-based vector.

It is further an object of the present invention to provide a method to produce transgenic animals through administration of a transposon-based vector that are capable of producing transgenic progeny.

Yet another object of the present invention is to provide a method to produce transgenic animals through administration of a transposon-based vector that are capable of producing a desired molecule, such as a protein, peptide or nucleic acid.

Another object of the present invention is to provide a method to produce transgenic animals through administration of a transposon-based vector, wherein such administration results in modulation of endogenous gene expression.

It is another object of the present invention to provide transposon-vectors useful for cell- or tissue-specific expression of a gene of interest in an animal or human with the purpose of gene therapy.

It is yet another object of the present invention to provide a method to produce transgenic avians through administration of a transposon-based vector that are capable of producing proteins, peptides or nucleic acids.

It is another object of the present invention to produce transgenic animals through administration of a transposon-based vector encoding an antibody or a fragment thereof.

Still another object of the present invention is to provide a method to produce transgenic avians through administration of a transposon-based vector that are capable of producing proteins or peptides and depositing these proteins or peptides in the egg.

Another object of the present invention is to provide transgenic avians that contain a stably incorporated transgene.

Still another object of the present invention is to provide eggs containing desired proteins or peptides encoded by a transgene incorporated into the transgenic avian that produces the egg.

It is further an object of the present invention to provide a method to produce transgenic milk-producing animals through administration of a transposon-based vector that are capable of producing proteins, peptides or nucleic acids.

Still another object of the present invention is to provide a method to produce transgenic milk-producing animals through administration of a transposon-based vector that are capable of producing proteins or peptides and depositing these proteins or peptides in their milk.

Another object of the present invention is to provide transgenic milk-producing animals that contain a stably incorporated transgene.

Another object of the present invention is to provide transgenic milk-producing animals that are capable of producing proteins or peptides and depositing these proteins or peptides in their milk.

Yet another object of the present invention is to provide milk containing desired molecules encoded by a transgene incorporated into the transgenic milk-producing animals that produce the milk.

Still another object of the present invention is to provide milk containing desired proteins or peptides encoded by a transgene incorporated into the transgenic milk-producing animals that produce the milk.

A further object of the present invention to provide a method to produce transgenic sperm through administration of a transposon-based vector to an animal.

A further object of the present invention to provide transgenic sperm that contain a stably incorporated transgene.

An advantage of the present invention is that transgenic animals are produced with higher efficiencies than observed in the prior art.

Another advantage of the present invention is that these transgenic animals possess high copy numbers of the transgene.

Another advantage of the present invention is that the transgenic animals produce large amounts of desired molecules encoded by the transgene.

Still another advantage of the present invention is that desired molecules are produced by the transgenic animals much more efficiently and economically than prior art methods, thereby providing a means for large scale production of desired molecules, particularly proteins and peptides.

Yet another advantage of the present invention is that the desired proteins and peptides are produced rapidly after making animals transgenic through introduction of the vectors of the present invention.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts schematically a transposon-based vector containing a transposase operably linked to a first promoter and a gene of interest operably-linked to a second promoter, wherein the gene of interest and its operably-linked promoter are flanked by insertion sequences (IS) recognized by the transposase. "Pro" designates a promoter. In this and subsequent figures, the size of the actual nucleotide sequence is not necessarily proportionate to the box representing that sequence.

Figure 2 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ov pro is the ovalbumin promoter, Ov protein is the ovalbumin protein and PolyA is a polyadenylation sequence. The TAG sequence includes a spacer sequence, the gp41 hairpin loop from HIV I and a protease cleavage site.

Figure 3 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg white wherein Ovo pro is the ovomucoid promoter and Ovo SS is the ovomucoid signal sequence. The TAG sequence includes a spacer, the gp41 hairpin loop from HIV I and a protease cleavage site.

Figure 4 depicts schematically a transposon-based vector for targeting deposition of a polypeptide in an egg yolk wherein Vit pro is the vitellogenin promoter and Vit targ is the vitellogenin targeting sequence.

Figure 5 depicts schematically a transposon-based vector for expression of antibody heavy and light chains. Prepro indicates a prepro sequence from cecropin and pro indicates a pro sequence from cecropin.

Figure 6 depicts schematically a transposon-based vector for expression of antibody heavy and light chains. Ent indicates an enterokinase cleavage sequence.

Figure 7 depicts schematically egg white targeted expression of antibody heavy and light chains from one vector in either tail-to-tail (Figure 7A) or tail-to-head (Figure 7B) configuration. In the tail-to-tail configuration, the ovalbumin signal sequence adjacent to the gene for the light chain contains on its 3' end an enterokinase cleavage site (not shown) to allow cleavage of the signal sequence from the light chain, and the ovalbumin signal sequence adjacent to the gene for the heavy chain contains on its 5' end an enterokinase cleavage site (not shown) to allow cleavage of the signal sequence from the heavy chain. In the tail-to-head configuration, the ovalbumin signal sequence adjacent to the gene for the heavy chain and the light chain contains on its 3' end an enterokinase cleavage site (not shown) to allow cleavage of the signal sequence from the heavy or light chain.

Figure 8 is a picture of an SDS-PAGE gel wherein a pooled fraction of an isolated proinsulin fusion protein was run in lanes 4 and 6. Lanes 1 and 10 of the gel contain molecular weight standards, lanes 2 and 8 contain non-trangenic chicken egg white, and lanes 3, 5, 7 and 9 are blank.

Figure 9 depicts schematically a transposon based-vector for expression of an RNAi molecule. "Tet_i pro" indicates a tetracycline inducible promoter whereas "pro" indicates the pro portion of a prepro sequence as described herein. "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferrin gene and "Ovomucin" indicates approximately 60 base pairs of an ovomucin gene.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new, effective and efficient method of producing transgenic animals, particularly egg-laying animals and milk-producing animals, through administration of a composition comprising a transposon-based vector designed for incorporation of a gene of interest and production of a desired molecule.

Definitions

It is to be understood that as used in the specification and in the claims, “a” or “an” can mean one or more, depending upon the context in which it is used. Thus, for example, reference to “a cell” can mean that at least one cell can be utilized.

The term “antibody” is used interchangeably with the term “immunoglobulin” and is defined herein as a protein synthesized by an animal or a cell of the immune system in response to the presence of a foreign substance commonly referred to as an “antigen” or an “immunogen”. The term antibody includes fragments of antibodies. Antibodies are characterized by specific affinity to a site on the antigen, wherein the site is referred to an “antigenic determinant” or an “epitope”. Antigens can be naturally occurring or artificially engineered. Artificially engineered antigens include, but are not limited to, small molecules, such as small peptides, attached to haptens such as macromolecules, for example proteins, nucleic acids, or polysaccharides. Artificially designed or engineered variants of naturally occurring antibodies and artificially designed or engineered antibodies not occurring in nature are all included in the current definition. Such variants include conservatively substituted amino acids and other forms of substitution as described in the section concerning proteins and polypeptides.

As used herein, the term “egg-laying animal” includes all amniotes such as birds, turtles, lizards and monotremes. Monotremes are egg-laying mammals and include the platypus and echidna. The term "bird" or "fowl," as used herein, is defined as a member of the Aves class of animals which are characterized as warm-blooded, egg-laying vertebrates primarily adapted for flying. Avians include, without limitation, Ratites, Psittaciformes, Falconiformes, Piciformes, Strigiformes, Passeriformes, Coraciformes, Ralliformes, Cuculiformes, Columbiformes, Galliformes, Anseriformes, and Herodiones. The term "Ratite," as used herein, is defined as a group of flightless, mostly large, running birds comprising several orders and including the emus, ostriches, kiwis, and cassowaries. The term "Psittaciformes", as used herein, includes parrots and refers to a monofamilial order of birds that exhibit zygodactylism and have a strong hooked bill. A "parrot" is defined as any member of the avian family Psittacidae (the single family of the Psittaciformes), distinguished by the short, stout, strongly hooked beak. Avians include all poultry birds, especially chickens, geese, turkeys, ducks and quail. The term “chicken” as used herein denotes

chickens used for table egg production, such as egg-type chickens, chickens reared for public meat consumption, or broilers, and chickens reared for both egg and meat production ("dual-purpose" chickens). The term "chicken" also denotes chickens produced by primary breeder companies, or chickens that are the parents, grandparents, great-grandparents, etc. of those chickens reared for public table egg, meat, or table egg and meat consumption.

The term "egg" is defined herein as including a large female sex cell enclosed in a porous, calcarous or leathery shell, produced by birds and reptiles. The term "ovum" is defined as a female gamete, and is also known as an egg. Therefore, egg production in all animals other than birds and reptiles, as used herein, is defined as the production and discharge of an ovum from an ovary, or "ovulation". Accordingly, it is to be understood that the term "egg" as used herein is defined as a large female sex cell enclosed in a porous, calcarous or leathery shell, when a bird or reptile produces it, or it is an ovum when it is produced by all other animals.

The term "milk-producing animal" refers herein to mammals including, but not limited to, bovine, ovine, porcine, equine, and primate animals. Milk-producing animals include but are not limited to cows, llamas, camels, goats, reindeer, zebu, water buffalo, yak, horses, pigs, rabbits, non-human primates, and humans.

The term "gene" is defined herein to include a coding region for a protein, peptide or polypeptide.

The term "transgenic animal" refers to an animal having at least a portion of the transposon-based vector DNA incorporated into its DNA. While a transgenic animal includes an animal wherein the transposon-based vector DNA is incorporated into the germline DNA, a transgenic animal also includes an animal having DNA in one or more cells that contain a portion of the transposon-based vector DNA for any period of time. In a preferred embodiment, a portion of the transposon-based vector comprises a gene of interest. More preferably, the gene of interest is incorporated into the animal's DNA for a period of at least five days, more preferably the reproductive life of the animal, and most preferably the life of the animal. In a further preferred embodiment, the animal is an avian.

The term "vector" is used interchangeably with the terms "construct", "DNA construct" and "genetic construct" to denote synthetic nucleotide sequences used for manipulation of genetic material, including but not limited to cloning, subcloning,

sequencing, or introduction of exogenous genetic material into cells, tissues or organisms, such as birds. It is understood by one skilled in the art that vectors may contain synthetic DNA sequences, naturally occurring DNA sequences, or both. The vectors of the present invention are transposon-based vectors as described herein.

When referring to two nucleotide sequences, one being a regulatory sequence, the term "operably-linked" is defined herein to mean that the two sequences are associated in a manner that allows the regulatory sequence to affect expression of the other nucleotide sequence. It is not required that the operably-linked sequences be directly adjacent to one another with no intervening sequence(s).

The term "regulatory sequence" is defined herein as including promoters, enhancers and other expression control elements such as polyadenylation sequences, matrix attachment sites, insulator regions for expression of multiple genes on a single construct, ribosome entry/attachment sites, introns that are able to enhance expression, and silencers.

Transposon-Based Vectors

While not wanting to be bound by the following statement, it is believed that the nature of the DNA construct is an important factor in successfully producing transgenic animals. The "standard" types of plasmid and viral vectors that have previously been almost universally used for transgenic work in all species, especially avians, have low efficiencies and may constitute a major reason for the low rates of transformation previously observed. The DNA (or RNA) constructs previously used often do not integrate into the host DNA, or integrate only at low frequencies. Other factors may have also played a part, such as poor entry of the vector into target cells. The present invention provides transposon-based vectors that can be administered to an animal that overcome the prior art problems relating to low transgene integration frequencies. Two preferred transposon-based vectors of the present invention in which a tranposase, gene of interest and other polynucleotide sequences may be introduced are termed pTnMCS (SEQ ID NO:2) and pTnMod (SEQ ID NO:3).

The transposon-based vectors of the present invention produce integration frequencies an order of magnitude greater than has been achieved with previous vectors. More specifically, intratesticular injections performed with a prior art transposon-based vector (described in U.S. Patent No. 5,719,055) resulted in 41% sperm positive roosters whereas intratesticular injections performed with the novel

transposon-based vectors of the present invention resulted in 77% sperm positive roosters. Actual frequencies of integration were estimated by either or both comparative strength of the PCR signal from the sperm and histological evaluation of the testes and sperm by quantitative PCR.

The transposon-based vectors of the present invention include a transposase gene operably-linked to a first promoter, and a coding sequence for a desired protein or peptide operably-linked to a second promoter, wherein the coding sequence for the desired protein or peptide and its operably-linked promoter are flanked by transposase insertion sequences recognized by the transposase. The transposon-based vector also includes one or more of the following characteristics: a) one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:1) at the 3' end of the first promoter to enhance expression of the transposase; b) modifications of the codons for the first several N-terminal amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) addition of an effective polyA sequence operably-linked to the transposase to further enhance expression of the transposase gene. The transposon-based vector may additionally or alternatively include one or more of the following Kozak sequence at the 3' end of any promoter, including the promoter operably-linked to the transposase: ACCATGG (SEQ ID NO:45), ACCATGT (SEQ ID NO:46), AAGATGT (SEQ ID NO:47), ACGATGA (SEQ ID NO:48), AAGATGG (SEQ ID NO:49), GACATGA (SEQ ID NO:50), ACCATGA (SEQ ID NO:51) and ACCATGA (SEQ ID NO:52).

Figure 1 shows a schematic representation of several components of the transposon-based vector. The present invention further includes vectors containing more than one gene of interest, wherein a second or subsequent gene of interest is operably-linked to the second promoter or to a different promoter. It is also to be understood that the transposon-based vectors shown in the Figures are representative of the present invention and that the order of the vector elements may be different than that shown in the Figures, that the elements may be present in various orientations, and that the vectors may contain additional elements not shown in the Figures.

Transposases and Insertion Sequences

In a further embodiment of the present invention, the transposase found in the transposase-based vector is an altered target site (ATS) transposase and the insertion sequences are those recognized by the ATS transposase. However, the transposase located in the transposase-based vectors is not limited to a modified ATS transposase and can be derived from any transposase. Transposases known in the prior art include those found in AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn 2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn10, Tn30, Tn101, Tn903, Tn501, Tn1000 ($\gamma\delta$), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons and I transposons. According to the present invention, these transposases and their regulatory sequences are modified for improved functioning as follows: a) the addition one or more modified Kozak sequences comprising ACCATG (SEQ ID NO:1) at the 3' end of the promoter operably-linked to the transposase; b) a change of the codons for the first several amino acids of the transposase, wherein the third base of each codon was changed to an A or a T without changing the corresponding amino acid; c) the addition of one or more stop codons to enhance the termination of transposase synthesis; and/or, d) the addition of an effective polyA sequencc operably-linked to the transposase to further enhance expression of the transposase gene.

Although not wanting to be bound by the following statement, it is believed that the modifications of the first several N-terminal codons of the transposase gene increase transcription of the transposase gene, in part, by increasing strand dissociation. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the transposase are modified such that the third base of each codon is changed to an A or a T without changing the encoded amino acid. In one embodiment, the first ten N-terminal codons of the transposase gene are modified in this manner. It is also preferred that the transposase contain mutations that make it less specific for preferred insertion sites and thus increases the rate of transgene insertion as discussed in U.S. Patent No. 5,719,055.

In some embodiments, the transposon-based vectors are optimized for expression in a particular host by changing the methylation patterns of the vector DNA. For example, prokaryotic methylation may be reduced by using a methylation

deficient organism for production of the transposon-based vector. The transposon-based vectors may also be methylated to resemble eukaryotic DNA for expression in a eukaryotic host.

Transposases and insertion sequences from other analogous eukaryotic transposon-based vectors that can also be modified and used are, for example, the Drosophila P element derived vectors disclosed in U.S. Patent No. 6,291,243; the Drosophila mariner element described in Sherman et al. (1998); or the sleeping beauty transposon. See also Hackett et al. (1999); D. Lampe et al., 1999. Proc. Natl. Acad. Sci. USA, 96:11428-11433; S. Fischer et al., 2001. Proc. Natl. Acad. Sci. USA, 98:6759-6764; L. Zagoraiou et al., 2001. Proc. Natl. Acad. Sci. USA, 98:11474-11478; and D. Berg et al. (Eds.), Mobile DNA, Amer. Soc. Microbiol. (Washington, D.C., 1989). However, it should be noted that bacterial transposon-based elements are preferred, as there is less likelihood that a eukaryotic transposase in the recipient species will recognize prokaryotic insertion sequences bracketing the transgene.

Many transposases recognize different insertion sequences, and therefore, it is to be understood that a transposase-based vector will contain insertion sequences recognized by the particular transposase also found in the transposase-based vector. In a preferred embodiment of the invention, the insertion sequences have been shortened to about 70 base pairs in length as compared to those found in wild-type transposons that typically contain insertion sequences of well over 100 base pairs.

While the examples provided below incorporate a "cut and insert" Tn10 based vector that is destroyed following the insertion event, the present invention also encompasses the use of a "rolling replication" type transposon-based vector. Use of a rolling replication type transposon allows multiple copies of the transposon/transgene to be made from a single transgene construct and the copies inserted. This type of transposon-based system thereby provides for insertion of multiple copies of a transgene into a single genome. A rolling replication type transposon-based vector may be preferred when the promoter operably-linked to gene of interest is endogenous to the host cell and present in a high copy number or highly expressed. However, use of a rolling replication system may require tight control to limit the insertion events to non-lethal levels. Tn1, Tn2, Tn3, Tn4, Tn5, Tn9, Tn21, Tn501, Tn551, Tn951, Tn1721, Tn2410 and Tn2603 are examples of a rolling replication type transposon, although Tn5 could be both a rolling replication and a cut and insert type transposon.

Stop Codons and PolyA Sequences

In one embodiment, the transposon-based vector contains two stop codons operably-linked to the transposase and/or to the gene of interest. In an alternate embodiment, one stop codon of UAA or UGA is operably linked to the transposase and/or to the gene of interest.

As used herein an "effective polyA sequence" refers to either a synthetic or non-synthetic sequence that contains multiple and sequential nucleotides containing an adenine base (an A polynucleotide string) and that increases expression of the gene to which it is operably-linked. A polyA sequence may be operably-linked to any gene in the transposon-based vector including, but not limited to, a transposase gene and a gene of interest. A preferred polyA sequence is optimized for use in the host animal or human. In one embodiment, the polyA sequence is optimized for use in an avian species, and more specifically, a chicken. An avian optimized polyA sequence generally contains a minimum of 40 base pairs, preferably between approximately 40 and several hundred base pairs, and more preferably approximately 75 base pairs that precede the A polynucleotide string and thereby separate the stop codon from the A polynucleotide string. In one embodiment of the present invention, thc polyA sequence comprises a conalbumin polyA sequence as provided in SEQ ID NO:4 and as taken from GenBank accession # Y00407, base pairs 10651-11058. In another embodiment, the polyA sequence comprises a synthetic polynucleotide sequence shown in SEQ ID NO:5. In yet another embodiment, the polyA sequence comprises an avian optimized polyA sequence provided in SEQ ID NO:6. A chicken optimized polyA sequence may also have a reduced amount of CT repeats as compared to a synthetic polyA sequence. In one embodiment of the present invention, the polyA sequence comprises a conalbumin polyA sequence as provided in SEQ ID NO:4 and as taken from GenBank accession # Y00407, base pairs 10651-11058.

It is a surprising discovery of the present invention that such an avian optimized poly A sequence increases expression of a polynucleotide to which it is operably-linked in an avian as compared to a non-avian optimized polyA sequence. Accordingly, the present invention includes methods of or increasing incorporation of a gene of interest wherein the gene of interest resides in a transposon-based vector containing a transposase gene and wherein the transposase gene is operably linked to an avian optimized polyA sequence. The present invention also includes methods of

increasing expression of a gene of interest in an avian that includes administering a gene of interest to the avian, wherein the gene of interest is operably-linked to an avian optimized polyA sequence. An avian optimized polyA nucleotide string is defined herein as a polynucleotide containing an A polynucleotide string and a minimum of 40 base pairs, preferably between approximately 40 and several hundred base pairs, and more preferably approximately 60 base pairs that precede the A polynucleotide string. The present invention further provides transposon-based vectors containing a gene of interest or transposase gene operably linked to an avian optimized polyA sequence.

Promoters and Enhancers

The first promoter operably-linked to the transposase gene and the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. Constitutive promoters include, but are not limited to, immediate early cytomegalovirus (CMV) promoter, herpes simplex virus 1 (HSV1) immediate early promoter, SV40 promoter, lysozyme promoter, early and late CMV promoters, early and late HSV promoters, β-actin promoter, tubulin promoter, Rous-Sarcoma virus (RSV) promoter, and heat-shock protein (HSP) promoter. Inducible promoters include tissue-specific promoters, developmentally-regulated promoters and chemically inducible promoters. Examples of tissue-specific promoters include the glucose 6 phosphate (G6P) promoter, vitellogenin promoter, ovalbumin promoter, ovomucoid promoter, conalbumin promoter, ovotransferrin promoter, prolactin promoter, kidney uromodulin promoter, and placental lactogen promoter. In one embodiment, the vitellogenin promoter includes a polynucleotide sequence of SEQ ID NO:7. The G6P promoter sequence may be deduced from a rat G6P gene untranslated upstream region provided in GenBank accession number U57552.1. Examples of developmentally-regulated promoters include the homeobox promoters and several hormone induced promoters. Examples of chemically inducible promoters include reproductive hormone induced promoters and antibiotic inducible promoters such as the tetracycline inducible promoter and the zinc-inducible metallothionine promoter.

Other inducible promoter systems include the Lac operator repressor system inducible by IPTG (isopropyl beta-D-thiogalactoside) (Cronin, A. et al. 2001. Genes and Development, v. 15), ecdysone-based inducible systems (Hoppe, U. C. et al.

2000. Mol. Ther. 1:159-164); estrogen-based inducible systems (Braselmann, S. et al. 1993. Proc. Natl. Acad. Sci. 90:1657-1661); progesterone-based inducible systems using a chimeric regulator, GLVP, which is a hybrid protein consisting of the GAL4 binding domain and the herpes simplex virus transcriptional activation domain, VP16, and a truncated form of the human progesterone receptor that retains the ability to bind ligand and can be turned on by RU486 (Wang, et al. 1994. Proc. Natl. Acad. Sci. 91:8180-8184); CID-based inducible systems using chemical inducers of dimerization (CIDs) to regulate gene expression, such as a system wherein rapamycin induces dimerization of the cellular proteins FKBP12 and FRAP (Belshaw, P. J. et al. 1996. J. Chem. Biol. 3:731-738; Fan, L. et al. 1999. Hum. Gene Ther. 10:2273-2285; Shariat, S.F. et al. 2001. Cancer Res. 61:2562-2571; Spencer, D.M. 1996. Curr. Biol. 6:839-847). Chemical substances that activate the chemically inducible promoters can be administered to the animal containing the transgene of interest via any method known to those of skill in the art.

Other examples of cell or tissue-specific and constitutive promoters include but are not limited to smooth-muscle SM22 promoter, including chimeric SM22alpha/telokin promoters (Hoggatt A.M. et al., 2002. Circ Res. 91(12):1151-9); ubiquitin C promoter (Biochim Biophys Acta, 2003. Jan. 3;1625(1):52-63); Hsf2 promoter; murine COMP (cartilage oligomeric matrix protein) promoter; early B cell-specific mb-1 promoter (Sigvardsson M., et al., 2002. Mol. Cell Biol. 22(24):8539-51); prostate specific antigen (PSA) promoter (Yoshimura I. et al., 2002, J. Urol. 168(6):2659-64); exorh promoter and pineal expression-promoting element (Asaoka Y., et al., 2002. Proc. Natl. Acad. Sci. 99(24):15456-61); neural and liver ceramidase gene promoters (Okino N. et al., 2002. Biochem. Biophys. Res. Commun. 299(1):160-6); PSP94 gene promoter/enhancer (Gabril M.Y. et al., 2002. Gene Ther. 9(23):1589-99); promoter of the human FAT/CD36 gene (Kuriki C., et al., 2002. Biol. Pharm. Bull. 25(11):1476-8); VL30 promoter (Staplin W.R. et al., 2002. Blood October 24, 2002); and, IL-10 promoter (Brenner S., et al., 2002. J. Biol. Chem. December 18, 2002).

Examples of avian promoters include, but are not limited to, promoters controlling expression of egg white proteins, such as ovalbumin, ovotransferrin (conalbumin), ovomucoid, lysozyme, ovomucin, g2 ovoglobulin, g3 ovoglobulin, ovoflavoprotein, ovostatin (ovomacroglobin), cystatin, avidin, thiamine-binding

protein, glutamyl aminopeptidase minor glycoprotein 1, minor glycoprotein 2; and promoters controlling expression of egg-yolk proteins, such as vitellogenin, very low-density lipoproteins, low density lipoprotein, cobalamin-binding protein, riboflavin-binding protein, biotin-binding protein (Awade, 1996. Z. Lebensm. Unters. Forsch. 202:1-14). An advantage of using the vitellogenin promoter is that it is active during the egg-laying stage of an animal's life-cycle, which allows for the production of the protein of interest to be temporally connected to the import of the protein of interest into the egg yolk when the protein of interest is equipped with an appropriate targeting sequence. In some embodiments, the avian promoter is an oviduct-specific promoter. As used herein, the term "oviduct-specific promoter" includes, but is not limited to, ovalbumin; ovotransferrin (conalbumin); ovomucoid; 01, 02, 03, 04 or 05 avidin; ovomucin; g2 ovoglobulin; g3 ovoglobulin; ovoflavoprotein; and ovostatin (ovomacroglobin) promoters.

Liver-specific promoters of the present invention include, but are not limited to, the following promoters, vitellogenin promoter, G6P promoter, cholesterol-7-alpha-hydroxylase (CYP7A) promoter, phenylalanine hydroxylase (PAH) promoter, protein C gene promoter, insulin-like growth factor I (IGF-I) promoter, bilirubin UDP-glucuronosyltransferase promoter, aldolase B promoter, furin promoter, metallothioneine promoter, albumin promoter, and insulin promoter.

Also included in the present invention are promoters that can be used to target expression of a protein of interest into the milk of a milk-producing animal including, but not limited to, β lactoglobin promoter, whey acidic protein promoter, lactalbumin promoter and casein promoter.

Promoters associated with cells of the immune system may also be used. Acute phase promoters such as interleukin (IL)-1 and IL-2 may be employed. Promoters for heavy and light chain Ig may also be employed. The promoters of the T cell receptor components CD4 and CD8, B cell promoters and the promoters of CR2 (complement receptor type 2) may also be employed. Immune system promoters are preferably used when the desired protein is an antibody protein.

Also included in this invention are modified promoters/enhancers wherein elements of a single promoter are duplicated, modified, or otherwise changed. In one embodiment, steroid hormone-binding domains of the ovalbumin promoter are moved from about -6.5 kb to within approximately the first 1000 base pairs of the gene of

interest. Modifying an existing promoter with promoter/enhancer elements not found naturally in the promoter, as well as building an entirely synthetic promoter, or drawing promoter/enhancer elements from various genes together on a non-natural backbone, are all encompassed by the current invention.

Accordingly, it is to be understood that the promoters contained within the transposon-based vectors of the present invention may be entire promoter sequences or fragments of promoter sequences. For example, in one embodiment, the promoter operably linked to a gene of interest is an approximately 900 base pair fragment of a chicken ovalbumin promoter (SEQ ID NO:8). The constitutive and inducible promoters contained within the transposon-based vectors may also be modified by the addition of one or more modified Kozak sequences of ACCATG (SEQ ID NO:1).

As indicated above, the present invention includes transposon-based vectors containing one or more enhancers. These enhancers may or may not be operably-linked to their native promoter and may be located at any distance from their operably-linked promoter. A promoter operably-linked to an enhancer and a promoter modified to eliminate repressive regulatory effects are referred to herein as an "enhanced promoter." The enhancers contained within the transposon-based vectors are preferably enhancers found in birds, and more preferably, an ovalbumin enhancer, but are not limited to these types of enhancers. In one embodiment, an approximately 675 base pair enhancer element of an ovalbumin promoter is cloned upstream of an ovalbumin promoter with 300 base pairs of spacer DNA separating the enhancer and promoter. In one embodiment, the enhancer used as a part of the present invention comprises base pairs 1-675 of a chicken ovalbumin enhancer from GenBank accession #S82527.1. The polynucleotide sequence of this enhancer is provided in SEQ ID NO:9.

Also included in some of the transposon-based vectors of the present invention are cap sites and fragments of cap sites. In one embodiment, approximately 50 base pairs of a 5' untranslated region wherein the capsite resides are added on the 3' end of an enhanced promoter or promoter. An exemplary 5' untranslated region is provided in SEQ ID NO:10. A putative cap-site residing in this 5' untranslated region preferably comprises the polynucleotide sequence provided in SEQ ID NO:11.

In one embodiment of the present invention, the first promoter operably-linked to the transposase gene is a constitutive promoter and the second promoter operably-

linked to the gene of interest is a tissue-specific promoter. In the second embodiment, use of the first constitutive promoter allows for constitutive activation of the transposase gene and incorporation of the gene of interest into virtually all cell types, including the germline of the recipient animal. Although the gene of interest is incorporated into the germline generally, the gene of interest may only be expressed in a tissue-specific manner. A transposon-based vector having a constitutive promoter operably-linked to the transposase gene can be administered by any route, and in one embodiment, the vector is administered to an ovary, to an artery leading to the ovary or to a lymphatic system or fluid proximal to the ovary.

It should be noted that cell- or tissue-specific expression as described herein does not require a complete absence of expression in cells or tissues other than the preferred cell or tissue. Instead, "cell-specific" or "tissue-specific" expression refers to a majority of the expression of a particular gene of interest in the preferred cell or tissue, respectively.

When incorporation of the gene of interest into the germline is not preferred, the first promoter operably-linked to the transposase gene can be a tissue-specific promoter. For example, transfection of a transposon-based vector containing a transposase gene operably-linked to a liver-specific promoter such as the G6P promoter or vitellogenin-promoter or vitellogenin promoter provides for activation of the transposase gene and incorporation of the gene of interest in the cells of the liver but not into the germline and other cells generally. In this embodiment, the second promoter operably-linked to the gene of interest can be a constitutive promoter or an inducible promoter. In a preferred embodiment, both the first promoter and the second promoter are a G6P promoter. In embodiments wherein tissue-specific expression or incorporation is desired, it is preferred that the transposon-based vector is administered directly to the tissue of interest or to an artery leading to the tissue of interest or to fluids surrounding the tissue of interest. In one embodiment, the tissue of interest is the oviduct and administration is achieved by direct injection into the oviduct or an artery leading to the oviduct. In a further preferred embodiment, administration is achieved by direct injection into the lumen of the magnum or the infundibulum of the oviduct. Indirect administration to the oviduct may occur through the cloaca.

Accordingly, cell specific promoters may be used to enhance transcription in selected tissues. In birds, for example, promoters that are found in cells of the fallopian tube, such as ovalbumin, conalbumin, ovomucoid and/or lysozyme, are used in the vectors to ensure transcription of the gene of interest in the epithelial cells and tubular gland cells of the fallopian tube, leading to synthesis of the desired protein encoded by the gene and deposition into the egg white. In mammals, promoters specific for the epithelial cells of the alveoli of the mammary gland, such as prolactin, insulin, beta lactoglobin, whey acidic protein, lactalbumin, casein, and/or placental lactogen, are used in the design of vectors used for transfection of these cells for the production of desired proteins for deposition into the milk. In liver cells, the G6P promoter may be employed to drive transcription of the gene of interest for protein production. Proteins made in the liver of birds may be delivered to the egg yolk.

In order to achieve higher or more efficient expression of the transposase gene, the promoter and other regulatory sequences operably-linked to the transposase gene may be those derived from the host. These host specific regulatory sequences can be tissue specific as described above or can be of a constitutive nature. For example, an avian actin promoter and its associated polyA sequcnce can be operably-linked to a transposase in a transposase-based vector for transfection into an avian. Examples of other host specific promoters that could be operably-linked to the transposase include the myosin and DNA or RNA polymerase promoters.

Directing Sequences

In some embodiments of the present invention, the gene of interest is operably-linked to a directing sequence or a sequence that provides proper conformation to the desired protein encoded by the gene of interest. As used herein, the term "directing sequence" refers to both signal sequences and targeting sequences. An egg directing sequence includes, but is not limited to, an ovomucoid signal sequence, an ovalbumin signal sequence, a cecropin pre pro signal sequence, and a vitellogenin targeting sequence. The term "signal sequence" refers to an amino acid sequence, or the polynucleotide sequence that encodes the amino acid sequence, that directs the protein to which it is linked to the endoplasmic reticulum in a eukaryote, and more preferably the translocational pores in the endoplasmic reticulum, or the plasma membrane in a prokaryote, or mitochondria, such as for the purpose of gene therapy for mitochondrial diseases. Signal and targeting sequences can be used to

direct a desired protein into, for example, the milk, when the transposon-based vectors are administered to a milk-producing animal.

Signal sequences can also be used to direct a desired protein into, for example, a secretory pathway for incorporation into the egg yolk or the egg white, when the transposon-based vectors are administered to a bird or other egg-laying animal. One example of such a transposon-based vector is provided in Figure 3 wherein the gene of interest is operably linked to the ovomucoid signal sequence. The present invention also includes a gene of interest operably-linked to a second gene containing a signal sequence. An example of such an embodiment is shown in Figure 2 wherein the gene of interest is operably-linked to the ovalbumin gene that contains an ovalbumin signal sequence. Other signal sequences that can be included in the transposon-based vectors include, but are not limited to the ovotransferrin and lysozyme signal sequences. In one embodiment, the signal sequence is an ovalbumin signal sequence including a sequence shown in SEQ ID NO:12. In another embodiment, the signal sequence is a modified ovalbumin signal sequence including a sequence shown in SEQ ID NO:13 or SEQ ID NO:14.

As also used herein, the term "targeting sequence" refers to an amino acid sequence, or the polynucleotide sequence encoding the amino acid sequence, which amino acid sequence is recognized by a receptor located on the exterior of a cell. Binding of the receptor to the targeting sequence results in uptake of the protein or peptide operably-linked to the targeting sequence by the cell. One example of a targeting sequence is a vitellogenin targeting sequence that is recognized by a vitellogenin receptor (or the low density lipoprotein receptor) on the exterior of an oocyte. In one embodiment, the vitellogenin targeting sequence includes the polynucleotide sequence of SEQ ID NO:15. In another embodiment, the vitellogenin targeting sequence includes all or part of the vitellogenin gene. Other targeting sequences include VLDL and Apo E, which are also capable of binding the vitellogenin receptor. Since the ApoE protein is not endogenously expressed in birds, its presence may be used advantageously to identify birds carrying the transposon-based vectors of the present invention.

Genes of Interest Encoding Desired Proteins

A gene of interest selected for stable incorporation is designed to encode any desired protein or peptide or to regulate any cellular response. In some embodiments,

the desired proteins or peptides are deposited in an egg or in milk. It is to be understood that the present invention encompasses transposon-based vectors containing multiple genes of interest. The multiple genes of interest may each be operably-linked to a separate promoter and other regulatory sequence(s) or may all be operably-linked to the same promoter and other regulatory sequences(s). In one embodiment, multiple genes of interest are linked to a single promoter and other regulatory sequence(s) and each gene of interest is separated by a cleavage site or a pro portion of a signal sequence. A gene of interest may contain modifications of the codons for the first several N-terminal amino acids of the gene of interest, wherein the third base of each codon is changed to an A or a T without changing the corresponding amino acid.

Protein and peptide hormones are a preferred class of proteins in the present invention. Such protein and peptide hormones are synthesized throughout the endocrine system and include, but are not limited to, hypothalamic hormones and hypophysiotropic hormones, anterior, intermediate and posterior pituitary hormones, pancreatic islet hormones, hormones made in the gastrointestinal system, renal hormones, thymic hormones, parathyroid hormones, adrenal cortical and medullary hormones. Specifically, hormones that can be produced using the present invention include, but are not limited to, chorionic gonadotropin, corticotropin, erythropoietin, IGF-1, oxytocin and its analogs, oxytocin receptor antagonist, platelet-derived growth factor, calcitonin, follicle-stimulating hormone, leutinizing hormone, thyroid-stimulating hormone, insulin, gonadotropin-releasing hormone and its analogs, gonadotropin-releasing hormone antagonist, vasopressin, octreotide, somatotrophin, somatostatin, prolactin, adrenocorticotropic hormone, antidiuretic hormone, thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), dopamine, melatonin, thyroxin (T_4), parathyroid hormone (PTH), glucocorticoids such as cortisol, mineralocorticoids such as aldosterone, androgens such as testosterone, adrenaline (epinephrine), noradrenaline (norepinephrine), estrogens such as estradiol, progesterone, glucagons, calcitrol, calciferol, atrial-natriuretic peptide, gastrin, secretin, cholecystokinin (CCK), neuropeptide Y, ghrelin, PYY_{3-36}, angiotensinogen, thrombopoietin, and leptin. By using appropriate polynucleotide sequences, species-specific hormones may be made by transgenic animals.

In one embodiment of the present invention, the gene of interest is a proinsulin gene and the desired molecule is insulin. Proinsulin consists of three parts: a C-peptide and two strands of amino acids (the alpha and beta chains) that later become linked together to form the insulin molecule. Figures 2 and 3 are schematics of transposon-based vector constructs containing a proinsulin gene operably-linked to an ovalbumin promoter and ovalbumin protein or an ovomucoid promoter and ovomucoid signal sequence, respectively. In these embodiments, proinsulin is expressed in the oviduct tubular gland cells and then deposited in the egg white. One example of a proinsulin polynucleotide sequence is shown in SEQ ID NO:16, wherein the C-peptide cleavage site spans from Arg at position 31 to Arg at position 65.

Serum proteins including lipoproteins such as high density lipoprotein (HDL), HDL-Milano and low density lipoprotein, albumin, clotting cascade factors, factor VIII, factor IX, fibrinogen, and globulins are also included in the group of desired proteins of the present invention. Immunoglobulins are one class of desired globulin molecules and include but are not limited to IgG, IgM, IgA, IgD, IgE, IgY, lambda chains, kappa chains and fragments thereof; Fc fragments, and Fab fragments. Desired antibodies include, but are not limited to, naturally occurring antibodies, human antibodies, humanized antibodies, and hybrid antibodies. Genes encoding modified versions of naturally occurring antibodies or fragments thereof and genes encoding artificially designed antibodies or fragments thereof may be incorporated into the transposon-based vectors of the present invention. Desired antibodies also include antibodies with the ability to bind specific ligands, for example, antibodies against proteins associated with cancer-related molecules, such as anti-her 2, or anti-CA125. Accordingly, the present invention encompasses a transposon-based vector containing one or more genes encoding a heavy immunoglobulin (Ig) chain and a light Ig chain. Further, more than one gene encoding for more than one antibody may be administered in one or more transposon-based vectors of the present invention. In this manner, an egg may contain more than one type of antibody in the egg white, the egg yolk or both. In one embodiment, a transposon-based vector contains a heavy Ig chain and a light Ig chain, both operably linked to a promoter. Figures 5 and 6 schematically depict exemplary constructs of this embodiment. More specifically, Figure 5 shows a construct containing a cecropin pre-pro sequence and a cecropin pro sequence, wherein the pre sequence functions to direct the resultant protein into the

endoplasmic reticulum and the pro sequences and the pro sequences are cleaved upon secretion of the protein from a cell into which the construct has been transfected. Figure 6 shows a construct containing an enterokinase cleavage site. In this embodiment, it may be required to further remove several additional amino acids from the light chain following cleavage by enterokinase. In another embodiment, the transposon-based vector comprises a heavy Ig chain operably-linked to one promoter and a light Ig chain operably-linked to another promoter. Figure 7 schematically depicts an exemplary construct of this embodiment. The present invention also encompasses a transposon-based vector containing genes encoding portions of a heavy Ig chain and/or portions of a light Ig chain. The present invention further includes a transposon-based vector containing a gene that encodes a fusion protein comprising a heavy and/or light Ig chain, or portions thereof.

Antibodies used as therapeutic reagents include but are not limited to antibodies for use in cancer immunotherapy against specific antigens, or for providing passive immunity to an animal or a human against an infectious disease or a toxic agent. Antibodies used as diagnostic reagents include, but are not limited to antibodies that may be labeled and detected with a detector, for example antibodies with a fluorescent label attached that may be detected following exposure to specific wavelengths. Such labeled antibodies may be primary antibodies directed to a specific antigen, for example, rhodamine-labeled rabbit anti-growth hormone, or may be labeled secondary antibodies, such as fluorescein-labeled goat-anti chicken IgG. Such labeled antibodies are known to one of ordinary skill in the art. Labels useful for attachment to antibodies are also known to one of ordinary skill in the art. Some of these labels are described in the "Handbook of Fluorescent Probes and Research Products", ninth edition, Richard P. Haugland (ed) Molecular Probes, Inc. Eugene, OR), which is incorporated herein in its entirety.

Antibodies produced with using the present invention may be used as laboratory reagents for numerous applications including radioimmunoassay, western blots, dot blots, ELISA, immunoaffinity columns and other procedures requiring antibodies as known to one of ordinary skill in the art. Such antibodies include primary antibodies, secondary antibodies and tertiary antibodies, which may be labeled or unlabeled.

Antibodies that may be made with the practice of the present invention include, but are not limited to primary antibodies, secondary antibodies, designer antibodies, anti-protein antibodies, anti-peptide antibodies, anti-DNA antibodies, anti-RNA antibodies, anti-hormone antibodies, anti-hypophysiotropic peptides, antibodies against non-natural antigens, anti-anterior pituitary hormone antibodies, anti-posterior pituitary hormone antibodies, anti-venom antibodies, anti-tumor marker antibodies, antibodies directed against epitopes associated with infectious disease, including, anti-viral, anti-bacterial, anti-protozoal, anti-fungal, anti-parasitic, anti-receptor, anti-lipid, anti-phospholipid, anti-growth factor, anti-cytokine, anti-monokine, anti-idiotype, and anti-accessory (presentation) protein antibodies. Antibodies made with the present invention, as well as light chains or heavy chains, may also be used to inhibit enzyme activity.

Antibodies that may be produced using the present invention include, but are not limited to, antibodies made against the following proteins: Bovine γ-Globulin, Serum; Bovine IgG, Plasma; Chicken γ-Globulin, Serum; Human γ-Globulin, Serum; Human IgA, Plasma; Human IgA_1, Myeloma; Human IgA_2, Myeloma; Human IgA_2, Plasma; Human IgD, Plasma; Human IgE, Myeloma; Human IgG, Plasma; Human IgG, Fab Fragment, Plasma; Human IgG, $F(ab')_2$ Fragment, Plasma; Human IgG, Fc Fragment, Plasma; Human IgG_1, Myeloma; Human IgG_2, Myeloma; Human IgG_3, Myeloma; Human IgG_4, Myeloma; Human IgM, Myeloma; Human IgM, Plasma; Human Immunoglobulin, Light Chain κ, Urine; Human Immunoglobulin, Light Chains κ and λ, Plasma; Mouse γ-Globulin, Serum; Mouse IgG, Serum; Mouse IgM, Myeloma; Rabbit γ-Globulin, Serum; Rabbit IgG, Plasma; and Rat γ-Globulin, Serum. In one embodiment, the transposon-based vector comprises the coding sequence of light and heavy chains of a murine monoclonal antibody that shows specificity for human seminoprotein (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

A further non-limiting list of antibodies that recognize other antibodies is as follows: Anti-Chicken IgG, heavy (H) & light (L) Chain Specific (Sheep); Anti-Goat γ-Globulin (Donkey); Anti-Goat IgG, Fc Fragment Specific (Rabbit); Anti-Guinea Pig γ-Globulin (Goat); Anti-Human Ig, Light Chain, Type κ Specific; Anti-Human Ig, Light Chain, Type λ Specific; Anti-Human IgA, α-Chain Specific (Goat); Anti-Human IgA, Fab Fragment Specific; Anti-Human IgA, Fc Fragment Specific; Anti-

Human IgA, Secretory; Anti-Human IgE, ϵ-Chain Specific (Goat); Anti-Human IgE, Fc Fragment Specific; Anti-Human IgG, Fc Fragment Specific (Goat); Anti-Human IgG, γ-Chain Specific (Goat); Anti-Human IgG, Fc Fragment Specific; Anti-Human IgG, Fd Fragment Specific; Anti-Human IgG, H & L Chain Specific (Goat); Anti-Human IgG_1, Fc Fragment Specific; Anti-Human IgG_2, Fc Fragment Specific; Anti-Human IgG_2, Fd Fragment Specific; Anti-Human IgG_3, Hinge Specific; Anti-Human IgG_4, Fc Fragment Specific; Anti-Human IgM, Fc Fragment Specific; Anti-Human IgM, μ-Chain Specific; Anti-Mouse IgE, ϵ-Chain Specific; Anti-Mouse γ-Globulin (Goat); Anti-Mouse IgG, γ-Chain Specific (Goat); Anti-Mouse IgG, γ-Chain Specific (Goat) $F(ab')_2$ Fragment; Anti-Mouse IgG, H & L Chain Specific (Goat); Anti-Mouse IgM, μ-Chain Specific (Goat); Anti-Mouse IgM, H & L Chain Specific (Goat); Anti-Rabbit γ-Globulin (Goat); Anti-Rabbit IgG, Fc Fragment Specific (Goat); Anti-Rabbit IgG, H & L Chain Specific (Goat); Anti-Rat γ-Globulin (Goat); Anti-Rat IgG, H & L Chain Specific; Anti-Rhesus Monkey γ-Globulin (Goat); and, Anti-Sheep IgG, H & L Chain Specific.

Another non-limiting list of the antibodies that may be produced using the present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. (www.phoenixpeptide.com, Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA, (St.Louis, MO www.sigma-aldrich.com), Cappel ICN (Irvine, California, www.icnbiomed.com), and Calbiochem, (La Jolla, CA, www.calbiochem.com), which are all incorporated herein by reference in their entirety. The polynucleotide sequences encoding these antibodies may be obtained from the scientific literature, from patents, and from databases such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing the codons that encode for each amino acid in the desired antibody. Antibodies made by the transgenic animals of the present invention include antibodies that may be used as therapeutic reagents, for example in cancer immunotherapy against specific antigens, as diagnostic reagents and as laboratory reagents for numerous applications including immunoneutralization, radioimmunoassay, western blots, dot blots, ELISA, immunoprecipitation and immunoaffinity columns. Some of these antibodies include, but are not limited to, antibodies which bind the following ligands: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory

peptide, gastrin releasing peptide, interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin, corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiostatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin, osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokinetican, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, prostaglandins and antagonist analogs, erythropoietin, p146 (SEQ ID NO:17 amino acid sequence, SEQ ID NO:18, nucleotide sequence), estrogen, testosterone, corticosteroids, mineralocorticoids, thyroid hormone, thymic hormones, connective tissue proteins, binding proteins, nuclear proteins, actin, avidin, activin, agrin, albumin, and prohormones, propeptides, splice variants, fragments and analogs thereof.

The following is yet another non-limiting list of antibodies that can be produced by the methods of present invention: abciximab (ReoPro), abciximab anti-platelet aggregation monoclonal antibody, anti-CD11a (hu1124), anti-CD18 antibody, anti-CD20 antibody, anti-cytomegalovirus (CMV) antibody, anti-digoxin antibody, anti-hepatitis B antibody, anti-HER-2 antibody, anti-idiotype antibody to GD3 glycolipid, anti-IgE antibody, anti-IL-2R antibody, antimetastatic cancer antibody (mAb 17-1A), anti-rabies antibody, anti-respiratory syncytial virus (RSV) antibody, anti-Rh antibody, anti-TCR, anti-TNF antibody, anti-VEGF antibody and fab fragment thereof, rattlesnake venom antibody, black widow spider venom antibody, coral snake venom antibody, antibody against very late antigen-4 (VLA-4), C225 humanized antibody to EGF receptor, chimeric (human & mouse) antibody against TNFα, antibody directed against GPIIb/IIIa receptor on human platelets, gamma

globulin, anti-hepatitis B immunoglobulin, human anti-D immunoglobulin, human antibodies against *S aureus*, human tetanus immunoglobulin, humanized antibody against the epidermal growth receptor-2, humanized antibody against the α subunit of the interleukin-2 receptor, humanized antibody CTLA4IG, humanized antibody to the IL-2 R α-chain, humanized anti-CD40-ligand monoclonal antibody (5c8), humanized mAb against the epidermal growth receptor-2, humanized mAb to rous sarcoma virus, humanized recombinant antibody (IgG1k) against respiratory syncytial virus (RSV), humanized (IgG1k) isotype mAb, lymphocyte immunoglobulin (anti-thymocyte antibody), lymphocyte immunoglobulin, mAb against factor VII, MDX-210 bi-specific antibody against HER-2, MDX-22, MDX-220 bi-specific antibody against TAG-72 on tumors, MDX-33 antibody to FcγR1 receptor, MDX-447 bi-specific antibody against EGF receptor, MDX-447 bispecific humanized antibody to EGF receptor, MDX-RA immunotoxin (ricin A linked) antibody, Medi-507 antibody (humanized form of BTI-322) against CD2 receptor on T-cells, monoclonal antibody LDP-02, muromonab-CD3(OKT3) antibody, OKT3 ("muromomab-CD3") antibody, PRO 542 antibody, ReoPro ("abciximab") antibody, TACI-Ig fusion protein, and TNF-IgG fusion protein.

The antibodies prepared using the methods of the present invention may also be designed to possess specific labels that may be detected through means known to one of ordinary skill in the art. The antibodies may also be designed to possess specific sequences useful for purification through means known to one of ordinary skill in the art. Specialty antibodies designed for binding specific antigens may also be made in transgenic animals using the transposon-based vectors of the present invention.

Production of a monoclonal antibody using the transposon-based vectors of the present invention can be accomplished in a variety of ways. In one embodiment, two vectors may be constructed: one that encodes the light chain, and a second vector that encodes the heavy chain of the monoclonal antibody. These vectors may then be incorporated into the genome of the target animal by methods disclosed herein. In an alternative embodiment, the sequences encoding light and heavy chains of a monoclonal antibody may be included on a single DNA construct. For example, the coding sequence of light and heavy chains of a murine monoclonal antibody that show specificity for human seminoprotein can be expressed using transposon-based

constructs of the present invention (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively).

Further included in the present invention are proteins and peptides synthesized by the immune system including those synthesized by the thymus, lymph nodes, spleen, and the gastrointestinal associated lymph tissues (GALT) system. The immune system proteins and peptides proteins that can be made in transgenic animals using the transposon-based vectors of the present invention include, but are not limited to, alpha-interferon, beta-interferon, gamma-interferon, alpha-interferon A, alpha-interferon 1, beta-interferon 1a, IFNAR-1, IFNAR-2, G-CSF, GM-CSF, interlukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-18, IL- binding proteins, TNF-α, TNF-β, and TNF binding proteins. Other cytokines included in the present invention include cardiotrophin, stromal cell derived factor, chemotactic cytokines, macrophage derived chemokine (MDC), melanoma growth stimulatory activity (MGSA), macrophage inflammatory proteins 1 alpha (MIP-1 alpha), 2, 3 alpha, 3 beta, 4 and 5.

Lytic peptides such as p146 are also included in the desired molecules of the present invention. In one embodiment, the p146 peptide comprises an amino acid sequence of SEQ ID NO:17. The present invention also encompasses a transposon-based vector comprising a p146 nucleic acid comprising a polynucleotide sequence of SEQ ID NO:18.

Enzymes are another class of proteins that may be made through the use of the transposon-based vectors of the present invention. Such enzymes include but are not limited to adenosine deaminase, alpha-galactosidase, cellulase, collagenase, dnaseI, hyaluronidase, lactase, L-asparaginase, pancreatin, papain, streptokinase B, subtilisin, superoxide dismutase, thrombin, trypsin, urokinase, fibrinolysin, glucocerebrosidase and plasminogen activator. In some embodiments wherein the enzyme could have deleterious effects, additional amino acids and a protease cleavage site are added to the carboxy end of the enzyme of interest in order to prevent expression of a functional enzyme. Subsequent digestion of the enzyme with a protease results in activation of the enzyme.

Extracellular matrix proteins are one class of desired proteins that may be made through the use of the present invention. Examples include but are not limited to collagen, fibrin, elastin, laminin, and fibronectin and subtypes thereof. Intracellular

proteins and structural proteins are other classes of desired proteins in the present invention.

Growth factors are another desired class of proteins that may be made through the use of the present invention and include, but are not limited to, transforming growth factor-α ("TGF-α"), transforming growth factor-β (TGF-β), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF), including FGF acidic isoforms 1 and 2, FGF basic form 2 and FGF 4, 8, 9 and 10, nerve growth factors (NGF) including NGF 2.5s, NGF 7.0s and beta NGF and neurotrophins, brain derived neurotrophic factor, cartilage derived factor, growth factors for stimulation of the production of red blood cells, growth factors for stimulation of the production of white blood cells, bone growth factors (BGF), basic fibroblast growth factor, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF), insulin like growth factor (IGF) I and II, hepatocyte growth factor, glial neurotrophic growth factor (GDNF), stem cell factor (SCF), keratinocyte growth factor (KGF), transforming growth factors (TGF), including TGFs alpha, beta, beta1, beta2, beta3, skeletal growth factor, bone matrix derived growth factors, bone derived growth factors, erythropoietin (EPO) and mixtures thereof.

Another desired class of proteins that may be made may be made through the use of the present invention include, but are not limited to, leptin, leukemia inhibitory factor (LIF), tumor necrosis factor alpha and beta, ENBREL, angiostatin, endostatin, thrombospondin, osteogenic protein-1, bone morphogenetic proteins 2 and 7, osteonectin, somatomedin-like peptide, and osteocalcin.

Yet another desired class of proteins are blood proteins or clotting cascade protein including albumin, Prekallikrein, High molecular weight kininogen (HMWK) (contact activation cofactor; Fitzgerald, Flaujeac Williams factor), Factor I (Fibrinogen), Factor II (prothrombin), Factor III (Tissue Factor), Factor IV (calcium), Factor V (proaccelerin, labile factor, accelerator (Ac-) globulin), Factor VI (Va) (accelerin), Factor VII (proconvertin), serum prothrombin conversion accelerator (SPCA), cothromboplastin), Factor VIII (antihemophiliac factor A, antihemophilic globulin (AHG)), Factor IX (Christmas Factor, antihemophilic factor B,plasma thromboplastin component (PTC)), Factor X (Stuart-Prower Factor), Factor XI (Plasma thromboplastin antecedent (PTA)), Factor XII (Hageman Factor), Factor XIII

(rotransglutaminase, fibrin stabilizing factor (FSF), fibrinoligase), von Willebrand factor, Protein C, Protein S, Thrombomodulin, Antithrombin III.

A non-limiting list of the peptides and proteins that may be made may be made through the use of the present invention is provided in product catalogs of companies such as Phoenix Pharmaceuticals, Inc. (www.phoenixpeptide.com; Belmont, CA), Peninsula Labs (San Carlos CA), SIGMA, (St.Louis, MO www.sigma-aldrich.com), Cappel ICN (Irvine, CA, www.icnbiomed.com), and Calbiochem (La Jolla, CA, www.calbiochem.com). The polynucleotide sequences encoding these proteins and peptides of interest may be obtained from the scientific literature, from patents, and from databases such as GenBank. Alternatively, one of ordinary skill in the art may design the polynucleotide sequence to be incorporated into the genome by choosing the codons that encode for each amino acid in the desired protein or peptide.

Some of these desired proteins or peptides that may be made through the use of the present invention include but are not limited to the following: adrenomedulin, amylin, calcitonin, amyloid, calcitonin gene-related peptide, cholecystokinin, gastrin, gastric inhibitory peptide, gastrin releasing peptide, interleukin, interferon, cortistatin, somatostatin, endothelin, sarafotoxin, glucagon, glucagon-like peptide, insulin, atrial natriuretic peptide, BNP, CNP, neurokinin, substance P, leptin, neuropeptide Y, melanin concentrating hormone, melanocyte stimulating hormone, orphanin, endorphin, dynorphin, enkephalin, leumorphin, peptide F, PACAP, PACAP-related peptide, parathyroid hormone, urocortin, corticotrophin releasing hormone, PHM, PHI, vasoactive intestinal polypeptide, secretin, ACTH, angiotensin, angiostatin, bombesin, endostatin, bradykinin, FMRF amide, galanin, gonadotropin releasing hormone (GnRH) associated peptide, GnRH, growth hormone releasing hormone, inhibin, granulocyte-macrophage colony stimulating factor (GM-CSF), motilin, neurotensin, oxytocin, vasopressin, osteocalcin, pancreastatin, pancreatic polypeptide, peptide YY, proopiomelanocortin, transforming growth factor, vascular endothelial growth factor, vesicular monoamine transporter, vesicular acetylcholine transporter, ghrelin, NPW, NPB, C3d, prokinetican, thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone, beta-lipotropin, melatonin, kallikriens, kinins, prostaglandins and antagonist analogs, erythropoietin, p146 (SEQ ID NO:17, amino acid sequence, SEQ ID NO:18, nucleotide sequence), thymic hormones, connective tissue proteins, binding proteins, beta sheet breaker

peptides, nuclear proteins, actin, avidin, activin, agrin, albumin, apolipoproteins, apolipoprotein A, apolipoprotein B, and prohormones, propeptides, splice variants, fragments and analogs thereof.

Other desired proteins that may be made by the transgenic animals of the present invention include bacitracin, polymixin b, vancomycin, cyclosporine, anti-RSV antibody, alpha-1 antitrypsin (AAT), anti-cytomegalovirus antibody, anti-hepatitis antibody, anti-inhibitor coagulant complex, anti-rabies antibody, anti-Rh(D) antibody, adenosine deaminase, anti-digoxin antibody, antivenin crotalidae (rattlesnake venom antibody), antivenin latrodectus (black widow spider venom antibody), antivenin micrurus (coral snake venom antibody), aprotinin, corticotropin (ACTH), diphtheria antitoxin, lymphocyte immune globulin (anti-thymocyte antibody), protamine, thyrotropin, capreomycin, α-galactosidase, gramicidin, streptokinase, tetanus toxoid, tyrothricin, IGF-1, proteins of varicella vaccine, anti-TNF antibody, anti-IL-2r antibody, anti-HER-2 antibody, OKT3 ("muromonab-CD3") antibody, TNF-IgG fusion protein, ReoPro ("abciximab") antibody, ACTH fragment 1-24, desmopressin, gonadotropin-releasing hormone, histrelin, leuprolide, lypressin, nafarelin, peptide that binds GPIIb/GPIIIa on platelets (integrilin), goserelin, capreomycin, colistin, anti-respiratory syncytial virus, lymphocyte immune globulin (Thymoglovin, Atgam), panorex, alpha-antitrypsin, botulinin, lung surfactant protein, tumor necrosis receptor-IgG fusion protein (enbrel), gonadorelin, proteins of influenza vaccine, proteins of rotavirus vaccine, proteins of haemophilus b conjugate vaccine, proteins of poliovirus vaccine, proteins of pneumococcal conjugate vaccine, proteins of meningococcal C vaccine, proteins of influenza vaccine, megakaryocyte growth and development factor (MGDF), neuroimmunophilin ligand-A (NIL-A), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), leptin (native), leptin B, leptin C, IL-1RA (interleukin-1RA), R-568, novel erythropoiesis-stimulating protein (NESP), humanized mAb to rous sarcoma virus (MEDI-493), glutamyl-tryptophan dipeptide IM862, LFA-3TIP immunosuppressive, humanized anti-CD40-ligand monoclonal antibody (5c8), gelsonin enzyme, tissue factor pathway inhibitor (TFPI), proteins of meningitis B vaccine, antimetastatic cancer antibody (mAb 17-1A), chimeric (human & mouse) mAb against TNFα, mAb against factor VII, relaxin, capreomycin, glycopeptide (LY333328), recombinant human activated protein C (rhAPC), humanized mAb against the epidermal growth

receptor-2, altepase, anti-CD20 antigen, C2B8 antibody, insulin-like growth factor-1, atrial natriuretic peptide (anaritide), tenectaplase, anti-CD11a antibody (hu 1124), anti-CD18 antibody, mAb LDP-02, anti-VEGF antibody, fab fragment of anti-VEGF Ab, APO2 ligand (tumor necrosis factor-related apoptosis-inducing ligand), rTGF-β (transforming growth factor-β), alpha-antitrypsin, ananain (a pineapple enzyme), humanized mAb CTLA4IG, PRO 542 (mAb), D2E7 (mAb), calf intestine alkaline phosphatase, α-L-iduronidase, α-L-galactosidase (humanglutamic acid decarboxylase, acid sphingomyelinase, bone morphogenetic protein-2 (rhBMP-2), proteins of HIV vaccine, T cell receptor (TCR) peptide vaccine, TCR peptides, V beta 3 and V beta 13.1. (IR502), (IR501), BI 1050/1272 mAb against very late antigen-4 (VLA-4), C225 humanized mAb to EGF receptor, anti-idiotype antibody to GD3 glycolipid, antibacterial peptide against *H. pylori*, MDX-447 bispecific humanized mAb to EGF receptor, anti-cytomegalovirus (CMV), Medi-491 B19 parvovirus vaccine, humanized recombinant mAb (IgG1k) against respiratory syncytial virus (RSV), urinary tract infection vaccine (against "pili" on *Escherechia coli* strains), proteins of lyme disease vaccine against *B. burgdorferi* protein (DbpA), proteins of Medi-501 human papilloma virus-11 vaccine (HPV), *Streptococcus pneumoniae* vaccine, Medi-507 mAb (humanized form of BTI-322) against CD2 receptor on T-cells, MDX-33 mAb to FcγR1 receptor, MDX-RA immunotoxin (ricin A linked) mAb, MDX-210 bi-specific mAb against HER-2, MDX-447 bi-specific mAb against EGF receptor, MDX-22, MDX-220 bi-specific mAb against TAG-72 on tumors, colony-stimulating factor (CSF) (molgramostim), humanized mAb to the IL-2 R α-chain (basiliximab), mAb to IgE (IGE 025A), myelin basic protein-altered peptide (MSP771A), humanized mAb against the epidermal growth receptor-2, humanized mAb against the α subunit of the interleukin-2 receptor, low molecular weight heparin, anti-hemophillic factor, and bactericidal/permeability-increasing protein (r-BPI).

The peptides and proteins made using the present invention may be labeled using labels and techniques known to one of ordinary skill in the art. Some of these labels are described in the "Handbook of Fluorescent Probes and Research Products", ninth edition, Richard P. Haugland (ed) Molecular Probes, Inc. Eugene, OR), which is incorporated herein in its entirety. Some of these labels may be genetically engineered into the polynucleotide sequence for the expression of the selected protein

or peptide. The peptides and proteins may also have label-incorporation "handles" incorporated to allow labeling of an otherwise difficult or impossible to label protein.

It is to be understood that the various classes of desired peptides and proteins, as well as specific peptides and proteins described in this section may be modified as described below by inserting selected codons for desired amino acid substitutions into the gene incorporated into the transgenic animal.

The present invention may also be used to produce desired molecules other than proteins and peptides including, but not limited to, lipoproteins such as high density lipoprotein (HDL), HDL-Milano, and low density lipoprotein, lipids, carbohydrates, siRNA and ribozymes. In these embodiments, a gene of interest encodes a nucleic acid molecule or a protein that directs production of the desired molecule.

The present invention further encompasses the use of inhibitory molecules to inhibit endogenous (i.e., non-vector) protein production. These inhibitory molecules include antisense nucleic acids, siRNA and inhibitory proteins. In a preferred embodiment, the endogenous protein whose expression is inhibited is an egg white protein including, but not limited to ovalbumin, ovotransferrin, and ovomucin. In one embodiment, a transposon-based vector containing an ovalbumin DNA sequence, that upon transcription forms a double stranded RNA molecule, is transfected into an animal such as a bird and the bird's production of endogenous ovalbumin protein is reduced by the interference RNA mechanism (RNAi). In other embodiments, a transposon-based vector encodes an inhibitory RNA molecule that inhibits the expression of more than one egg white protein. One exemplary construct is provided in Figure 9 wherein "Ovgen" indicates approximately 60 base pairs of an ovalbumin gene, "Ovotrans" indicates approximately 60 base pairs of an ovotransferrin gene and "Ovomucin" indicates approximately 60 base pairs of an ovomucin gene. These ovalbumin, ovotransferrin and ovomucin can be from any avian species, and in some embodiments, are from a chicken or quail. The term "pro" indicates the pro portion of a prepro sequence. One exemplary prepro sequence is that of cecropin and comprising base pairs 563-733 of the Cecropin cap site and prepro provided in Genbank accession number X07404. Additional cecropin prepro and pro sequences are provided in SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, and SEQ ID NO:44, respectively. Additionally, inducible knockouts or knockdowns of the endogenous

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protein may be created to achieve a reduction or inhibition of endogenous protein production. Endogenous egg white production can be inhibited in an avian at any time, but is preferably inhibited preceding, or immediately preceding, the harvest of eggs.

Modified Desired Proteins and Peptides

"Proteins", "peptides," "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (i.e., the amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (i.e., the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the protein, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the protein. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a protein, or to the carboxyl group of an amino acid at any other location within the protein.

Typically, the amino acids making up a protein are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the protein. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the protein than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (i.e., amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill will recognize that, as mentioned above, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than about 5%, more typically less than about 1%) in an encoded sequence are conservatively modified variations where

the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

A conservative substitution is a substitution in which the substituting amino acid (naturally occurring or modified) is structurally related to the amino acid being substituted, i.e., has about the same size and electronic properties as the amino acid being substituted. Thus, the substituting amino acid would have the same or a similar functional group in the side chain as the original amino acid. A "conservative substitution" also refers to utilizing a substituting amino acid which is identical to the amino acid being substituted except that a functional group in the side chain is protected with a suitable protecting group.

Suitable protecting groups are described in Green and Wuts, "Protecting Groups in Organic Synthesis", John Wiley and Sons, Chapters 5 and 7, 1991, the teachings of which are incorporated herein by reference. Preferred protecting groups are those which facilitate transport of the peptide through membranes, for example, by reducing the hydrophilicity and increasing the lipophilicity of the peptide, and which can be cleaved, either by hydrolysis or enzymatically (Ditter et al., 1968. J. Pharm. Sci. 57:783; Ditter et al., 1968. J. Pharm. Sci. 57:828; Ditter et al., 1969. J. Pharm. Sci. 58:557; King et al., 1987. Biochemistry 26:2294; Lindberg et al., 1989. Drug Metabolism and Disposition 17:311; Tunek et al., 1988. Biochem. Pharm. 37:3867; Anderson et al., 1985 Arch. Biochem. Biophys. 239:538; and Singhal et al., 1987. FASEB J. 1:220). Suitable hydroxyl protecting groups include ester, carbonate and carbamate protecting groups. Suitable amine protecting groups include acyl groups and alkoxy or aryloxy carbonyl groups, as described above for N-terminal protecting groups. Suitable carboxylic acid protecting groups include aliphatic, benzyl and aryl esters, as described below for C-terminal protecting groups. In one embodiment, the

carboxylic acid group in the side chain of one or more glutamic acid or aspartic acid residues in a peptide of the present invention is protected, preferably as a methyl, ethyl, benzyl or substituted benzyl ester, more preferably as a benzyl ester.

Provided below are groups of naturally occurring and modified amino acids in which each amino acid in a group has similar electronic and steric properties. Thus, a conservative substitution can be made by substituting an amino acid with another amino acid from the same group. It is to be understood that these groups are non-limiting, i.e. that there are additional modified amino acids which could be included in each group.

Group I includes leucine, isoleucine, valine, methionine and modified amino acids having the following side chains: ethyl, n-propyl n-butyl. Preferably, Group I includes leucine, isoleucine, valine and methionine.

Group II includes glycine, alanine, valine and a modified amino acid having an ethyl side chain. Preferably, Group II includes glycine and alanine.

Group III includes phenylalanine, phenylglycine, tyrosine, tryptophan, cyclohexylmethyl glycine, and modified amino residues having substituted benzyl or phenyl side chains. Preferred substituents include one or more of the following: halogen, methyl, ethyl, nitro, —NH_2, methoxy, ethoxy and —CN. Preferably, Group III includes phenylalanine, tyrosine and tryptophan.

Group IV includes glutamic acid, aspartic acid, a substituted or unsubstituted aliphatic, aromatic or benzylic ester of glutamic or aspartic acid (e.g., methyl, ethyl, n-propyl iso-propyl, cyclohexyl, benzyl or substituted benzyl), glutamine, asparagine, —CO—NH— alkylated glutamine or asparagines (e.g., methyl, ethyl, n-propyl and iso-propyl) and modified amino acids having the side chain —$(CH_2)_3$—COOH, an ester thereof (substituted or unsubstituted aliphatic, aromatic or benzylic ester), an amide thereof and a substituted or unsubstituted N-alkylated amide thereof. Preferably, Group IV includes glutamic acid, aspartic acid, methyl aspartate, ethyl aspartate, benzyl aspartate and methyl glutamate, ethyl glutamate and benzyl glutamate, glutamine and asparagine.

Group V includes histidine, lysine, ornithine, arginine, N-nitroarginine, ß-cycloarginine, γ-hydroxyarginine, N-amidinocitruline and 2-amino-4-guanidinobutanoic acid, homologs of lysine, homologs of arginine and

homologs of ornithine. Preferably, Group V includes histidine, lysine, arginine and ornithine. A homolog of an amino acid includes from 1 to about 3 additional or subtracted methylene units in the side chain.

Group VI includes serine, threonine, cysteine and modified amino acids having C1-C5 straight or branched alkyl side chains substituted with —OH or —SH, for example, —CH_2CH_2OH, —$CH_2CH_2CH_2OH$ or -$CH_2CH_2OHCH_3$. Preferably, Group VI includes serine, cysteine or threonine.

In another aspect, suitable substitutions for amino acid residues include "severe" substitutions. A "severe substitution" is a substitution in which the substituting amino acid (naturally occurring or modified) has significantly different size and/or electronic properties compared with the amino acid being substituted. Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of severe substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for glycine, a D amino acid for the corresponding L amino acid, or —NH—CH[(—$CH_2)_5$—COOH]—CO— for aspartic acid. Alternatively, a functional group may be added to the side chain, deleted from the side chain or exchanged with another functional group. Examples of severe substitutions of this type include adding of valine, leucine or isoleucine, exchanging the carboxylic acid in the side chain of aspartic acid or glutamic acid with an amine, or deleting the amine group in the side chain of lysine or ornithine. In yet another alternative, the side chain of the substituting amino acid can have significantly different steric and electronic properties that the functional group of the amino acid being substituted. Examples of such modifications include tryptophan for glycine, lysine for aspartic acid and —$(CH_2)_4COOH$ for the side chain of serine. These examples are not meant to be limiting.

In another embodiment, for example in the synthesis of a peptide 26 amino acids in length, the individual amino acids may be substituted according in the following manner:

AA_1 is serine, glycine, alanine, cysteine or threonine;

AA_2 is alanine, threonine, glycine, cysteine or serine;

AA_3 is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, ß-cycloarginine, γ-hydroxyarginine, N-amidinocitruline or 2-amino-4-guanidinobutanoic acid;

AA_4 is proline, leucine, valine, isoleucine or methionine;

AA_5 is tryptophan, alanine, phenylalanine, tyrosine or glycine;

AA_6 is serine, glycine, alanine, cysteine or threonine;

AA_7 is proline, leucine, valine, isoleucine or methionine;

AA_8 is alanine, threonine, glycine, cysteine or serine;

AA_9 is alanine, threonine, glycine, cysteine or serine;

AA_{10} is leucine, isoleucine, methionine or valine;

AA_{11} is serine, glycine, alanine, cysteine or threonine;

AA_{12} is leucine, isoleucine, methionine or valine;

AA_{13} is leucine, isoleucine, methionine or valine;

AA_{14} is glutamine, glutamic acid, aspartic acid, asparagine, or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;

AA_{15} is arginine, N-nitroarginine, ß-cycloarginine, γ-hydroxy-arginine, N-amidinocitruline or 2-amino-4-guanidino-butanoic acid

AA_{16} is proline, leucine, valine, isoleucine or methionine;

AA_{17} is serine, glycine, alanine, cysteine or threonine;

AA_{18} is glutamic acid, aspartic acid, asparagine, glutamine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;

AA_{19} is aspartic acid, asparagine, glutamic acid, glutamine, leucine, valine, isoleucine, methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;

AA_{20} is valine, arginine, leucine, isoleucine, methionine, ornithine, lysine, N-nitroarginine, ß-cycloarginine, γ-hydroxyarginine, N-amidinocitruline or 2-amino-4-guanidinobutanoic acid;

AA_{21} is alanine, threonine, glycine, cysteine or serine;

AA_{22} is alanine, threonine, glycine, cysteine or serine;

AA_{23} is histidine, serine, threonine, cysteine, lysine or ornithine;

AA_{24} is threonine, aspartic acid, serine, glutamic acid or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid;

AA_{25} is asparagine, aspartic acid,, glutamic acid, glutamine, leucine, valine, isoleucine, methionine or a substituted or unsubstituted aliphatic or aryl ester of glutamic acid or aspartic acid; and

AA_{26} is cysteine, histidine, serine, threonine, lysine or ornithine.

It is to be understood that these amino acid substitutions may be made for longer or shorter peptides than the 26 mer in the preceding example above, and for proteins.

In one embodiment of the present invention, codons for the first several N-terminal amino acids of the transposase are modified such that the third base of each codon is changed to an A or a T without changing the corresponding amino acid. It is preferable that between approximately 1 and 20, more preferably 3 and 15, and most preferably between 4 and 12 of the first N-terminal codons of the gene of interest are modified such that the third base of each codon is changed to an A or a T without changing the corresponding amino acid. In one embodiment, the first ten N-terminal codons of the gene of interest are modified in this manner.

When several desired proteins, protein fragments or peptides are encoded in the gene of interest to be incorporated into the genome, one of skill in the art will appreciate that the proteins, protein fragments or peptides may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino acids. Generally, the spacer will have no specific biological activity other than to join the desired proteins, protein fragments or peptides together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. The spacer may also be contained within a nucleotide sequence with a purification handle or be flanked by cleavage sites, such as proteolytic cleavage sites.

Such polypeptide spacers may have from about 5 to about 40 amino acid residues. The spacers in a polypeptide are independently chosen, but are preferably all the same. The spacers should allow for flexibility of movement in space and are therefore typically rich in small amino acids, for example, glycine, serine, proline or alanine. Preferably, peptide spacers contain at least 60%, more preferably at least 80% glycine or alanine. In addition, peptide spacers generally have little or no biological and antigenic activity. Preferred spacers are $(\text{Gly-Pro-Gly-Gly})_x$ (SEQ ID

NO:19) and (Gly$_4$-Ser)$_y$, wherein x is an integer from about 3 to about 9 and y is an integer from about 1 to about 8. Specific examples of suitable spacers include

(Gly-Pro-Gly-Gly)$_3$

SEQ ID NO:20 Gly Pro Gly Gly Gly Pro Gly Gly Gly Pro Gly Gly

(Gly$_4$-Ser)$_3$

SEQ ID NO:21 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser

or (Gly$_4$-Ser)$_4$

SEQ ID NO:22 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser.

Nucleotide sequences encoding for the production of residues which may be useful in purification of the expressed recombinant protein may also be built into the vector. Such sequences are known in the art and include the glutathione binding domain from glutathione S-transferase, polylysine, hexa-histidine or other cationic amino acids, thioredoxin, hemagglutinin antigen and maltose binding protein.

Additionally, nucleotide sequences may be inserted into the gene of interest to be incorporated so that the protein or peptide can also include from one to about six amino acids that create signals for proteolytic cleavage. In this manner, if a gene is designed to make one or more peptides or proteins of interest in the transgenic animal, specific nucleotide sequences encoding for amino acids recognized by enzymes may be incorporated into the gene to facilitate cleavage of the large protein or peptide sequence into desired peptides or proteins or both. For example, nucleotides encoding a proteolytic cleavage site can be introduced into the gene of interest so that a signal sequence can be cleaved from a protein or peptide encoded by the gene of interest. Nucleotide sequences encoding other amino acid sequences which display pH sensitivity or chemical sensitivity may also be added to the vector to facilitate separation of the signal sequence from the peptide or protein of interest.

Proteolytic cleavage sites include cleavage sites recognized by exopeptidases such as carboxypeptidase A, carboxypeptidase B, aminopeptidase I, and dipeptidylaminopeptidase; endopeptidases such as trypsin, V8-protease, enterokinase, factor Xa, collagenase, endoproteinase, subtilisin, and thombin; and proteases such as Protease 3C IgA protease (Igase) Rhinovirus 3C(preScission)protease. Chemical cleavage sites are also included in the defintion of cleavage site as used herein.

Chemical cleavage sites include, but are not limited to, site cleaved by cyanogen bromide, hydroxylamine, formic acid, and acetic acid.

In one embodiment of the present invention, a TAG sequence is linked to the gene of interest. The TAG sequence serves three purposes: 1) it allows free rotation of the peptide or protein to be isolated so there is no interference from the native protein or signal sequence, i.e. vitellogenin, 2) it provides a "purification handle" to isolate the protein using column purification, and 3) it includes a cleavage site to remove the desired protein from the signal and purification sequences. Accordingly, as used herein, a TAG sequence includes a spacer sequence, a purification handle and a cleavage site. The spacer sequences in the TAG proteins contain one or more repeats shown in SEQ ID NO:23. A preferred spacer sequence comprises the sequence provided in SEQ ID NO:24. One example of a purification handle is the gp41 hairpin loop from HIV I. Exemplary gp41 polynucleotide and polypeptide sequences are provided in SEQ ID NO:25 and SEQ ID NO:26, respectively. However, it should be understood that any antigenic region may be used as a purification handle, including any antigenic region of gp41. Preferred purification handles are those that elicit highly specific antibodies. Additionally, the cleavage site can be any protein cleavage site known to one of ordinary skill in the art and includes an enterokinase cleavage site comprising the Asp Asp Asp Asp Lys sequence (SEQ ID NO:27) and a furin cleavage site. Constructs containing a TAG sequence are shown in Figures 2 and 3. In one embodiment of the present invention, the TAG sequence comprises a polynucleotide sequence of SEQ ID NO:28.

Methods of Administering Transposon-Based Vectors

In addition to the transposon-based vectors described above, the present invention also includes methods of administering the transposon-based vectors to an animal, methods of producing a transgenic animal wherein a gene of interest is incorporated into the germline of the animal and methods of producing a transgenic animal wherein a gene of interest is incorporated into cells other than the germline cells (somatic cells) of the animal. The transposon-based vectors of the present invention may be administered to an animal via any method known to those of skill in the art, including, but not limited to, intraembryonic, intratesticular, intraoviduct, intraovarian, intraperitoneal, intraarterial, intravenous, topical, oral, nasal, and pronuclear injection methods of administration, or any combination thereof. The

transposon-based vectors may also be administered within the lumen of an organ, into an organ, into a body cavity, into the cerebrospinal fluid, through the urinary system or through any route to reach the desired cells.

In one embodiment the transposon-based vectors are administered to a reproductive organ including, but not limited to, an oviduct, an ovary, or into the duct system of the mammary gland. The vectors may be directly administered to a reproductive organ or can be administered to an artery leading to the reproductive organ or to a lymph system proximate to the cells to be genetically altered. The vectors may be administered to a reproductive organ of an animal through the cloaca. One method of direct administration is by injection, and in one embodiment, the lumen of the magnum of the oviduct is injected with a transposon-based vector. Another method of direct administration is by injection, and in one embodiment, the lumen of the infundibulum of the oviduct is injected with a transposon-based vector. A preferred intrarterial administration is an administration into an artery that supplies the oviduct or the ovary. In some embodiments, administration of the transposon-based vector to an oviduct or an artery that leads to the oviduct results in incorporation of the vector into the epithelial and/or secretory cells of the oviduct. In other embodiments, administration of the transposon-based vector to an ovary or an artery that leads to the ovary or a lymphatic system proximal to the ovary results in incorporation of the vector into an oocyte or a germinal disk inside the ovary.

The transposon-based vectors may be delivered through the vascular system to be distributed to the cells supplied by that vessel. For example, the vectors may additionally or alternatively be placed in the artery supplying the ovary or supplying the fallopian tube to transfect cells in those tissues. In this manner, follicles could be transfected to create a germline transgenic animal. Alternatively, supplying the compositions through the artery leading to the oviduct would preferably transfect the tubular gland and epithelial cells. Such transfected cells could manufacture a desired protein or peptide for deposition in the egg white. Administration of transposon-based vectors may occur in arteries supplying the ovary and or through direct intrathecal administration into the ovary through injection. Administration of the compositions through the portal vein would target uptake and transformation of hepatic cells. Administration through the urethra and into the bladder would target the transitional epithelium of the bladder. Administration through the vagina and

cervix would target the lining of the uterus. Administration through the internal mammary artery would transfect secretory cells of the lactating mammary gland to perform a desired function, such as to synthesize and secrete a desired protein or peptide into the milk. Direct administration into the mammary gland comprises introduction into the duct system of the mammary gland.

The transposon-based vectors may be administered in a single administration, multiple administrations, continuously, or intermittently. The transposon-based vectors may be administered by injection, via a catheter, an osmotic mini-pump or any other method. In some embodiments, the transposon-based vector is administered to an animal in multiple administrations, each administration containing the vector and a different transfecting reagent.

The transposon-based vectors may be administered to the animal at any point during the lifetime of the animal, however, it is preferable that the vectors are administered prior to the animal reaching sexual maturity. The transposon-based vectors are preferably administered to a chicken between approximately 14 and 16 weeks of age and to a quail between approximately 5 and 6 weeks of age when standard poultry rearing practices are used. The vectors may be administered at earlier ages when exogenous hormones are used to induce early sexual maturation in the bird. In some embodiments, the transposon-based vector is administered to an animal following an increase in proliferation of the oviduct epithelial cells and/or the tubular gland cells. Such an increase in proliferation normally follows an influx of reproductive hormones in the area of the oviduct. When the animal is an avian, the transposon-based vector is administered following an increase in proliferation of the oviduct epithelial cells and before the avian begins to produce egg white constituents.

In a preferred embodiment, the animal is an egg-laying animal, and more preferably, an avian. In one embodiment, between approximately 1 and 150 μg, 1 and 100 μg, 1 and 50 μg, preferably between 1 and 20 μg, and more preferably between 5 and 10 μg of transposon-based vector DNA is administered to the oviduct of a bird. Optimal ranges depend upon the type of bird and the bird's stage of sexual maturity. In a chicken, it is preferred that between approximately 1 and 100 μg, or 5 and 50 μg are administered. In a quail, it is preferred that between approximately 5 and 10 μg are administered. Intraoviduct administration of the transposon-based vectors of the present invention result in incorporation of the gene of interest into the cells of the

oviduct as evidenced by a PCR positive signal in the oviduct tissue, whereas intravascular administration results in incorporation of the gene of interest into the cells of the liver as evidence by a PCR positive signal in the liver. In other embodiments, the transposon-based vector is administered to an artery that supplies the oviduct or the liver. These methods of administration may also be combined with any methods for facilitating transfection, including without limitation, electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

The present invention includes a method of intraembryonic administration of a transposon-based vector to an avian embryo comprising the following steps: 1) incubating an egg on its side at room temperature for two hours to allow the embryo contained therein to move to top dead center (TDC); 2) drilling a hole through the shell without penetrating the underlying shell membrane; 3) injecting the embryo with the transposon-based vector in solution; 4) sealing the hole in the egg; and 5) placing the egg in an incubator for hatching. Administration of the transposon-based vector can occur anytime between immediately after egg lay (when the embryo is at Stage X) and hatching. Preferably, the transposon-based vector is administered between 1 and 7 days after egg lay, more preferably between 1 and 2 days after egg lay. The transposon-based vectors may be introduced into the embryo in amounts ranging from about 5.0 μg to 10 pg, preferably 1.0 μg to 100 pg. Additionally, the transposon-based vector solution volume may be between approximately 1 μl to 75 μl in quail and between approximately 1 μl to 500 μl in chicken.

The present invention also includes a method of intratesticular administration of a transposon-based vector including injecting a bird with a composition comprising the transposon-based vector, an appropriate carrier and an appropriate transfection reagent. In one embodiment, the bird is injected before sexual maturity, preferably between approximately 4-14 weeks, more preferably between approximately 6-14 weeks and most preferably between 8-12 weeks old. In another embodiment, a mature bird is injected with a transposon-based vector an appropriate carrier and an appropriate transfection reagent. The mature bird may be any type of bird, but in one example the mature bird is a quail.

A bird is preferably injected prior to the development of the blood-testis barrier, which thereby facilitates entry of the transposon-based vector into the seminiferous tubules and transfection of the spermatogonia or other germline cells.

At and between the ages of 4, 6, 8, 10, 12, and 14 weeks, it is believed that the testes of chickens are likely to be most receptive to transfection. In this age range, the blood/testis barrier has not yet formed, and there is a relatively high number of spermatogonia relative to the numbers of other cell types, e.g., spermatids, etc. See J. Kumaran et al., 1949. Poultry Sci., 29:511-520. See also E. Oakberg, 1956. Am. J. Anatomy, 99:507-515; and P. Kluin et al., 1984. Anat. Embryol., 169:73-78.

The transposon-based vectors may be introduced into a testis in an amount ranging from about 0.1 µg to 10 µg, preferably 1 µg to 10 µg, more preferably 3 µg to 10 µg. In a quail, about 5 µg is a preferred amount. In a chicken, about 5 µg to 10 µg per testis is preferred. These amounts of vector DNA may be injected in one dose or multiple doses and at one site or multiple sites in the testis. In a preferred embodiment, the vector DNA is administered at multiple sites in a single testis, both testes being injected in this manner. In one embodiment, injection is spread over three injection sites: one at each end of the testis, and one in the middle. Additionally, the transposon-based vector solution volume may be between approximately 1 µl to 75 µl in quail and between approximately 1 µl to 500 µl in chicken. In a preferred embodiment, the transposon-based vector solution volume may be between approximately 20 µl to 60 µl in quail and between approximately 50 µl to 250 µl in chicken. Both the amount of vector DNA and the total volume injected into each testis may be determined based upon the age and size of the bird.

According to the present invention, the transposon-based vector is administered in conjunction with an acceptable carrier and/or transfection reagent. Acceptable carriers include, but are not limited to, water, saline, Hanks Balanced Salt Solution (HBSS), Tris-EDTA (TE) and lyotropic liquid crystals. Transfection reagents commonly known to one of ordinary skill in the art that may be employed include, but are not limited to, the following: cationic lipid transfection reagents, cationic lipid mixtures, polyamine reagents, liposomes and combinations thereof; SUPERFECT®, Cytofectene, BioPORTER®, GenePORTER®, NeuroPORTER®, and perfectin from Gene Therapy Systems; lipofectamine, cellfectin, DMRIE-C oligofectamine, TROJENE® and PLUS reagent from InVitrogen; Xtreme gene, fugene, DOSPER and DOTAP from Roche; Lipotaxi and Genejammer from Strategene; and Escort from SIGMA. In one embodiment, the transfection reagent is SUPERFECT®. The ratio of DNA to transfection reagent may vary based upon the

method of administration. In one embodiment, the transposon-based vector is administered intratesticularly and the ratio of DNA to transfection reagent can be from 1:1.5 to 1:15, preferably 1:2 to 1:10, all expressed as wt/vol. Transfection may also be accomplished using other means known to one of ordinary skill in the art, including without limitation electroporation, gene guns, injection of naked DNA, and use of dimethyl sulfoxide (DMSO).

Depending upon the cell or tissue type targeted for transfection, the form of the transposon-based vector may be important. Plasmids harvested from bacteria are generally closed circular supercoiled molecules, and this is the preferred state of a vector for gene delivery because of the ease of preparation. In some instances, transposase expression and insertion may be more efficient in a relaxed, closed circular configuration or in a linear configuration. In still other instances, a purified transposase protein may be co-injected with a transposon-based vector containing the gene of interest for more immediate insertion. This could be accomplished by using a transfection reagent complexed with both the purified transposase protein and the transposon-based vector.

Testing for and Breeding Animals Carrying the Transgene

Following administration of a transposon-based vector to an animal, DNA is extracted from the animal to confirm integration of the gene of interest. Advantages provided by the present invention include the high rates of integration, or incorporation, and transcription of the gene of interest when administered to a bird.

Actual frequencies of integration may be estimated both by comparative strength of the PCR signal, and by histological evaluation of the tissues by quantitative PCR. Another method for estimating the rate of transgene insertion is the so-called primed in situ hybridization technique (PRINS). This method determines not only which cells carry a transgene of interest, but also into which chromosome the gene has inserted, and even what portion of the chromosome. Briefly, labeled primers are annealed to chromosome spreads (affixed to glass slides) through one round of PCR, and the slides are then developed through normal in situ hybridization procedures. This technique combines the best features of in situ PCR and fluorescence in situ hybridization (FISH) to provide distinct chromosome location and copy number of the gene in question. The 28s rRNA gene will be used as a positive control for spermatogonia to confirm that the technique is functioning properly.

Using different fluorescent labels for the transgene and the 28s gene causes cells containing a transgene to fluoresce with two different colored tags.

Breeding experiments are also conducted to determine if germline transmission of the transgene has occurred. In a general bird breeding experiment performed according to the present invention, each male bird was exposed to 2-3 different adult female birds for 3-4 days each. This procedure was continued with different females for a total period of 6-12 weeks. Eggs are collected daily for up to 14 days after the last exposure to the transgenic male, and each egg was incubated in a standard incubator. In the first series of experiments the resulting embryos were examined for transgene presence at day 3 or 4 using PCR.

Any male producing a transgenic embryo was bred to additional females. Eggs from these females were incubated, hatched, and the chicks tested for the exogenous DNA. Any embryos that died were necropsied and examined directly for the transgene or protein encoded by the transgene, either by fluorescence or PCR. The offspring that hatched and were found to be positive for the exogenous DNA were raised to maturity. These birds were bred to produce further generations of transgenic birds, to verify efficiency of the transgenic procedure and the stable incorporation of the transgene into the germ line. The resulting embryos are examined for transgene presence at day 3 or 4 using PCR. It is to be understood that the above procedure can be modified to suit animals other than birds and that selective breeding techniques may be performed to amplify gene copy numbers and protein output.

Production of Desired Proteins or Peptides in Egg White

In one embodiment, the transposon-based vectors of the present invention may be administered to a bird for production of desired proteins or peptides in the egg white. These transposon-based vectors preferably contain one or more of an ovalbumin promoter, an ovomucoid promoter, an ovalbumin signal sequence and an ovomucoid signal sequence. Oviduct-specific ovalbumin promoters are described in B. O'Malley et al., 1987. EMBO J., vol. 6, pp. 2305-12; A. Qiu et al., 1994. Proc. Nat. Acad. Sci. (USA), vol. 91, pp. 4451-4455; D. Monroe et al., 2000. Biochim. Biophys. Acta, 1517 (1):27-32; H. Park et al., 2000. Biochem., 39:8537-8545; and T. Muramatsu et al., 1996. Poult. Avian Biol. Rev., 6:107-123. Examples of transposon-

based vectors designed for production of a desired protein in an egg white are shown in Figures 2 and 3.

Production of Desired Proteins or Peptides in Egg Yolk

The present invention is particularly advantageous for production of recombinant peptides and proteins of low solubility in the egg yolk. Such proteins include, but are not limited to, membrane-associated or membrane-bound proteins, lipophilic compounds; attachment factors, receptors, and components of second messenger transduction machinery. Low solubility peptides and proteins are particularly challenging to produce using conventional recombinant protein production techniques (cell and tissue cultures) because they aggregate in water-based, hydrophilic environments. Such aggregation necessitates denaturation and re-folding of the recombinantly-produced proteins, which may deleteriously affect their structure and function. Moreover, even highly soluble recombinant peptides and proteins may precipitate and require denaturation and renaturation when produced in sufficiently high amounts in recombinant protein production systems. The present invention provides an advantageous resolution of the problem of protein and peptide solubility during production of large amounts of recombinant proteins.

In one embodiment of the present invention, deposition of a desired protein into the egg yolk is accomplished in offspring by attaching a sequence encoding a protein capable of binding to the yolk vitellogenin receptor to a gene of interest that encodes a desired protein. This transposon-based vector can be used for the receptor-mediated uptake of the desired protein by the oocytes. In a preferred embodiment, the sequence ensuring the binding to the vitellogenin receptor is a targeting sequence of a vitellogenin protein. The invention encompasses various vitellogenin proteins and their targeting sequences. In a preferred embodiment, a chicken vitellogenin protein targeting sequence is used, however, due to the high degree of conservation among vitellogenin protein sequences and known cross-species reactivity of vitellogenin targeting sequences with their egg-yolk receptors, other vitellogenin targeting sequences can be substituted. One example of a construct for use in the transposon-based vectors of the present invention and for deposition of an insulin protein in an egg yolk is provided in SEQ ID NO:53.

In this embodiment the, a transposon-based vector containing a vitellogenin promoter, a vitellogenin targeting sequence, a TAG sequence, a pro-insulin sequence

and a synthetic polyA sequence. The present invention includes, but is not limited to, vitellogenin targeting sequences residing in the N-terminal domain of vitellogenin, particularly in lipovitellin I. In one embodiment, the vitellogenin targeting sequence contains the polynucleotide sequence of SEQ ID NO:15. In a preferred embodiment, the transposon-based vector contains a transposase gene operably-linked to a liver-specific promoter and a gene of interest operably-linked to a liver-specific promoter and a vitellogenin targeting sequence. Figure 4 shows an example of such a construct. In another preferred embodiment, the transposon-based vector contains a transposase gene operably-linked to a constitutive promoter and a gene of interest operably-linked to a liver-specific promoter and a vitellogenin targeting sequence.

Isolation and Purification of Desired Protein or Peptide

For large-scale production of protein, an animal breeding stock that is homozygous for the transgene is preferred. Such homozygous individuals are obtained and identified through, for example, standard animal breeding procedures or PCR protocols.

Once expressed, peptides, polypeptides and proteins can be purified according to standard procedures known to one of ordinary skill in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis, high performance liquid chromatography, immunoprecipitation and the like. Substantially pure compositions of about 50 to 99% homogeneity are preferred, and 80 to 95% or greater homogeneity are most preferred for use as therapeutic agents.

In one embodiment of the present invention, the animal in which the desired protein is produced is an egg-laying animal. In a preferred embodiment of the present invention, the animal is an avian and a desired peptide, polypeptide or protein is isolated from an egg white. Egg white containing the exogenous protein or peptide is separated from the yolk and other egg constituents on an industrial scale by any of a variety of methods known in the egg industry. See, e.g., W. Stadelman et al. (Eds.), Egg Science & Technology, Haworth Press, Binghamton, NY (1995). Isolation of the exogenous peptide or protein from the other egg white constituents is accomplished by any of a number of polypeptide isolation and purification methods well known to one of ordinary skill in the art. These techniques include, for example, chromatographic methods such as gel permeation, ion exchange, affinity separation, metal chelation, HPLC, and the like, either alone or in combination. Another means

that may be used for isolation or purification, either in lieu of or in addition to chromatographic separation methods, includes electrophoresis. Successful isolation and purification is confirmed by standard analytic techniques, including HPLC, mass spectroscopy, and spectrophotometry. These separation methods are often facilitated if the first step in the separation is the removal of the endogenous ovalbumin fraction of egg white, as doing so will reduce the total protein content to be further purified by about 50%.

To facilitate or enable purification of a desired protein or peptide, transposon-based vectors may include one or more additional epitopes or domains. Such epitopes or domains include DNA sequences encoding enzymatic or chemical cleavage sites including, but not limited to, an enterokinase cleavage site; the glutathione binding domain from glutathione S-transferase; polylysine; hexa-histidine or other cationic amino acids; thioredoxin; hemagglutinin antigen; maltose binding protein; a fragment of gp41 from HIV; and other purification epitopes or domains commonly known to one of skill in the art.

In one representative embodiment, purification of desired proteins from egg white utilizes the antigenicity of the ovalbumin carrier protein and particular attributes of a TAG linker sequence that spans ovalbumin and the desired protein. The TAG sequence is particularly useful in this process because it contains 1) a highly antigenic epitope, a fragment of gp41 from HIV, allowing for stringent affinity purification, and, 2) a recognition site for the protease enterokinase immediately juxtaposed to the desired protein. In a preferred embodiment, the TAG sequence comprises approximately 50 amino acids. A representative TAG sequence is provided below.

Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp <u>Ala Thr Thr Cys Ile Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu</u> *Asp Asp Asp Asp Lys* (SEQ ID NO:28)

The underlined sequences were taken from the hairpin loop domain of HIV gp-41 (SEQ ID NO:25). Sequences in italics represent the cleavage site for enterokinase (SEQ ID NO:27). The spacer sequence upstream of the loop domain was made from repeats of (Pro Ala Asp Asp Ala) (SEQ ID NO:24) to provide free rotation and promote surface availability of the hairpin loop from the ovalbumin carrier protein.

Isolation and purification of a desired protein is performed as follows:

1. Enrichment of the egg white protein fraction containing ovalbumin and the transgenic ovalbumin-TAG-desired protein.
2. Size exclusion chromatography to isolate only those proteins within a narrow range of molecular weights (a further enrichment of step 1).
3. Ovalbumin affinity chromatography. Highly specific antibodies to ovalbumin will eliminate virtually all extraneous egg white proteins except ovalbumin and the transgenic ovalbumin-TAG-desired protein.
4. gp41 affinity chromatography using anti-gp41 antibodies. Stringent application of this step will result in virtually pure transgenic ovalbumin-TAG-desired protein.
5. Cleavage of the transgene product can be accomplished in at least one of two ways:
    a. The transgenic ovalbumin-TAG-desired protein is left attached to the gp41 affinity resin (beads) from step 4 and the protease enterokinase is added. This liberates the transgene target protein from the gp41 affinity resin while the ovalbumin-TAG sequence is retained. Separation by centrifugation (in a batch process) or flow through (in a column purification), leaves the desired protein together with enterokinase in solution. Enterokinase is recovered and reused.
    b. Alternatively, enterokinase is immobilized on resin (beads) by the addition of poly-lysine moieties to a non-catalytic area of the protease. The transgenic ovalbumin-TAG-desired protein eluted from the affinity column of step 4 is then applied to the protease resin. Protease action cleaves the ovalbumin-TAG sequence from the desired protein and leaves both entities in solution. The immobilized enterokinase resin is recharged and reused.
    c. The choice of these alternatives is made depending upon the size and chemical composition of the transgene target protein.
6. A final separation of either of these two (5a or 5b) protein mixtures is made using size exclusion, or enterokinase affinity chromatography. This step allows for desalting, buffer exchange and/or polishing, as needed.

Cleavage of the transgene product (ovalbumin-TAG-desired protein) by enterokinase, then, results in two products: ovalbumin-TAG and the desired protein. More specific methods for isolation using the TAG label is provided in the Examples. Some desired proteins may require additions or modifications of the above-described approach as known to one of ordinary skill in the art. The method is scaleable from the laboratory bench to pilot and production facility largely because the techniques applied are well documented in each of these settings.

It is believed that a typical chicken egg produced by a transgenic animal of the present invention will contain at least 0.001 mg, from about 0.001 to 1.0 mg, or from about 0.001 to 100.0 mg of exogenous protein, peptide or polypeptide, in addition to the normal constituents of egg white (or possibly replacing a small fraction of the latter).

One of skill in the art will recognize that after biological expression or purification, the desired proteins, fragments thereof and peptides may possess a conformation substantially different than the native conformations of the proteins, fragments thereof and peptides. In this case, it is often necessary to denature and reduce protein and then to cause the protein to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art.

Production of Protein or Peptide in Milk

In addition to methods of producing eggs containing transgenic proteins or peptides, the present invention encompasses methods for the production of milk containing transgenic proteins or peptides. These methods include the administration of a transposon-based vector described above to a mammal through the duct system. In one embodiment, the transposon-based vector contains a transposase operably-linked to a constitutive promoter and a gene of interest operably-linked to mammary specific promoter. Genes of interest can include, but are not limited to antiviral and antibacterial proteins and immunoglobulins.

Treatment of Disease and Animal Improvement

In addition to production and isolation of desired molecules, the transposon-based vectors of the present invention can be used for the treatment of various genetic disorders. For example, one or more transposon-based vectors can be administered to a human or animal for the treatment of a single gene disorder including, but not

limited to, Huntington's disease, alpha-1-antitrypsin deficiency, Alzheimer's disease, various forms or breast cancer, cystic fibrosis, galactosemia, congenital hypothyroidism, maple syrup urine disease, neurofibromatosis 1, phenylketonuria, sickle cell disease, and Smith-Lemli-Opitz (SLO/RSH) Syndrome. Other diseases caused by single gene disorders that may be treated with the present invention include, autoimmune diseases, shipping fever in cattle, mastitis, bacterial or viral diseases, alteration of skin pigment in animals. In these embodiments, the transposon-based vector contains a non-mutated, or non-disease causing form of the gene known to cause such disorder. Preferably, the transposase contained within the transposase-based vector is operably linked to an inducible promoter such as a tissue-specific promoter such that the non-mutated gene of interest is inserted into a specific tissue wherein the mutated gene is expressed in vivo.

In one embodiment of the present invention, a transposon-based vector comprising a gene encoding proinsulin is administered to diabetic animals or humans for incorporation into liver cells in order to treat or cure diabetes. The specific incorporation of the proinsulin gene into the liver is accomplished by placing the transposase gene under the control of liver-specific promoter, such as G6P. This approach is useful for treatment of both Type I and Type II diabetes. The G6P promoter has been shown to be glucose responsive (Arguad, D., et al. 1996. Diabetes 45:1563-1571), and thus, glucose-regulated insulin production is achieved using DNA constructs of the present invention. Integrating a proinsulin gene into liver cells circumvents the problem of destruction of pancreatic islet cells in the course of Type I diabetes.

In another embodiment, shortly after diagnosis of Type I diabetes, the cells of the immune system destroying pancreatic β-cells are selectively removed using the transposon-based vectors of the present invention, thus allowing normal β-cells to repopulate the pancreas.

For treatment of Type II diabetes, a transposon-based vector containing a proinsulin gene is specifically incorporated into the pancreas by placing the transposase gene under the control of a pancreas-specific promoter, such as an insulin promoter. In this embodiment, the vector is delivered to a diabetic animal or human via injection into an artery feeding the pancreas. For delivery, the vector is complexed with a transfection agent. The artery distributes the complex throughout

the pancreas, where individual cells receive the vector DNA. Following uptake into the target cell, the insulin promoter is recognized by transcriptional machinery of the cell, the transposase encoded by the vector is expressed, and stable integration of the proinsulin gene occurs. It is expected that a small percentage of the transposon-based vector is transported to other tissues, and that these tissues are transfected. However, these tissues are not stably transfected and the proinsulin gene is not incorporated into the cells' DNA due to failure of these cells to activate the insulin promoter. The vector DNA is likely lost when the cell dies or degraded over time.

In other embodiments, one or more transposon-based vectors are administered to an avian for the treatment of a viral or bacterial infection/disease including, but not limited to, Colibacillosis (Coliform infections), Mycoplasmosis (CRD, Air sac, Sinusitis), Fowl Cholera, Necrotic Enteritis, Ulcerative Enteritis (Quail disease), Pullorum Disease, Fowl Typhoid, Botulism, Infectious Coryza, Erysipelas, Avian Pox, Newcastle Disease, Infectious Bronchitis, Quail Bronchitis, Lymphoid Leukosis, Marek's Disease (Visceral Leukosis), Infectious Bursal Disease (Gumboro). In these embodiments, the transposon-based vectors may be used in a manner similar to traditional vaccines.

In still other embodiments, one or more transposon-based vectors are administered to an animal for the production of an animal with enhanced growth characteristics and nutrient utilization.

The transposon-based vectors of the present invention can be used to transform any animal cell, including but not limited to: cells producing hormones, cytokines, growth factors, or any other biologically active substance; cells of the immune system; cells of the nervous system; muscle (striatal, cardiac, smooth) cells; vascular system cells; endothelial cells; skin cells; mammary cells; and lung cells, including bronchial and alveolar cells. Transformation of any endocrine cell by a transposon-based vector is contemplated as a part of a present invention. In one aspect of the present invention, cells of the immune system may be the target for incorporation of a desired gene or genes encoding for production of antibodies. Accordingly, the thymus, bone marrow, beta lymphocytes (or B cells), gastrointestinal associated lymphatic tissue (GALT), Peyer's patches, bursa Fabricius, lymph nodes, spleen, and tonsil, and any other lymphatic tissue, may all be targets for administration of the compositions of the present invention.

The transposon-based vectors of the present invention can be used to modulate (stimulate or inhibit) production of any substance, including but not limited to a hormone, a cytokine, an enzyme, or a growth factor, by an animal or a human cell. Modulation of a regulated signal within a cell or a tissue, such as production of a second messenger, is also contemplated as a part of the present invention. Use of the transposon-based vectors of the present invention is contemplated for treatment of any animal or human disease or condition that results from underproduction (such as diabetes) or overproduction (such as hyperthyroidism) of a hormone or other endogenous biologically active substance. Use of the transposon-based vectors of the present invention to integrate nucleotide sequences encoding RNA molecules, such as anti-sense RNA or short interfering RNA, is also contemplated as a part of the present invention.

Additionally, the transposon-based vectors of the present invention may be used to provide cells or tissues with "beacons", such as receptor molecules, for binding of therapeutic agents in order to provide tissue and cell specificity for the therapeutic agents. Several promoters and exogenous genes can be combined in one vector to produce progressive, controlled treatments from a single vector delivery.

The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various embodiments, modifications and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the invention.

EXAMPLE 1

Preparation of Transposon-Based Vector pTnMod

A vector was designed for inserting a desired coding sequence into the genome of eukaryotic cells, given below as SEQ ID NO:3. The vector of SEQ ID NO:3, termed pTnMod, was constructed and its sequence verified.

This vector employed a cytomegalovirus (CMV) promoter. A modified Kozak sequence (ACCATG) (SEQ ID NO:1) was added to the promoter. The nucleotide in the wobble position in nucleotide triplet codons encoding the first 10 amino acids of transposase was changed to an adenine (A) or thymine (T), which did not alter the

amino acid encoded by this codon. Two stop codons were added and a synthetic polyA was used to provide a strong termination sequence. This vector uses a promoter designed to be active soon after entering the cell (without any induction) to increase the likelihood of stable integration. The additional stop codons and synthetic polyA insures proper termination without read through to potential genes downstream.

The first step in constructing this vector was to modify the transposase to have the desired changes. Modifications to the transposase were accomplished with the primers High Efficiency forward primer (Hef) Altered transposase (ATS)-Hef 5' ATCTCGAGACCATGTGTGAACTTGATATTTACATGATTCTCTTTACC 3' (SEQ ID NO:29) and Altered transposase- High efficiency reverse primer (Her) 5' GATTGATCATTATCATAATTTCCCCAAAGCGTAACC 3' (SEQ ID NO:30, a reverse complement primer). In the 5' forward primer ATS-Hef, the sequence CTCGAG (SEQ ID NO:31) is the recognition site for the restriction enzyme Xho I, which permits directional cloning of the amplified gene. The sequence ACCATG (SEQ ID NO:1) contains the Kozak sequence and start codon for the transposase and the underlined bases represent changes in the wobble position to an A or T of codons for the first 10 amino acids (without changing the amino acid coded by the codon). Primer ATS-Her (SEQ ID NO:30) contains an additional stop codon TAA in addition to native stop codon TGA and adds a Bcl I restriction site, TGATCA (SEQ ID NO:32), to allow directional cloning. These primers were used in a PCR reaction with pTnLac (p defines plasmid, tn defines transposon, and lac defines the beta fragment of the lactose gene, which contains a multiple cloning site) as the template for the transposase and a FailSafe™ PCR System (which includes enzyme, buffers, dNTP's, $MgCl_2$ and PCR Enhancer; Epicentre Technologies, Madison, WI). Amplified PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. A band corresponding to the expected size was excised from the gel and purified from the agarose using a Zymo Clean Gel Recovery Kit (Zymo Research, Orange, CA). Purified DNA was digested with restriction enzymes Xho I (5') and Bcl I (3') (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Digested DNA was purified from restriction enzymes using a Zymo DNA Clean and Concentrator kit (Zymo Research).

Plasmid gWhiz (Gene Therapy Systems, San Diego, CA) was digested with restriction enzymes Sal I and BamH I (New England Biolabs), which are compatible with Xho I and Bcl I, but destroy the restriction sites. Digested gWhiz was separated on an agarose gel, the desired band excised and purified as described above. Cutting the vector in this manner facilitated directional cloning of the modified transposase (mATS) between the CMV promoter and synthetic polyA.

To insert the mATS between the CMV promoter and synthetic polyA in gWhiz, a Stratagene T4 Ligase Kit (Stratagene, Inc. La Jolla, CA) was used and the ligation set up according to the manufacturer's protocol. Ligated product was transformed into *E. coli* Top10 competent cells (Invitrogen Life Technologies, Carlsbad, CA) using chemical transformation according to Invitrogen's protocol. Transformed bacteria were incubated in 1 ml of SOC (GIBCO BRL, CAT# 15544-042) medium for 1 hour at 37° C before being spread to LB (Luria-Bertani media (broth or agar)) plates supplemented with 100 μg/ml ampicillin (LB/amp plates). These plates were incubated overnight at 37° C and resulting colonies picked to LB/amp broth for overnight growth at 37° C. Plasmid DNA was isolated using a modified alkaline lysis protocol (Sambrook et al., 1989), electrophoresed on a 1% agarose gel, and visualized on a U.V. transilluminator after ethidium bromide staining. Colonies producing a plasmid of the expected size (approximately 6.4 kbp) were cultured in at least 250 ml of LB/amp broth and plasmid DNA harvested using a Qiagen Maxi-Prep Kit (column purification) according to the manufacturer's protocol (Qiagen, Inc., Chatsworth, CA). Column purified DNA was used as template for sequencing to verify the changes made in the transposase were the desired changes and no further changes or mutations occurred due to PCR amplification. For sequencing, Perkin-Elmer's Big Dye Sequencing Kit was used. All samples were sent to the Gene Probes and Expression Laboratory (LSU School of Veterinary Medicine) for sequencing on a Perkin-Elmer Model 377 Automated Sequencer.

Once a clone was identified that contained the desired mATS in the correct orientation, primers CMVf-NgoM IV (5' TTGCCGGCATCAGATTGGCTAT (SEQ ID NO:33); underlined bases denote a NgoM IV recognition site) and Syn-polyA-BstE II (5' AGAGGTCACCGGGTCAATTCTTCAGCACCTGGTA (SEQ ID NO:34); underlined bases denote a BstE II recognition site) were used to PCR amplify the entire CMV promoter, mATS, and synthetic polyA for cloning upstream of the

ATLLIB01 1625871.3

transposon in pTnLac. The PCR was conducted with FailSafe™ as described above, purified using the Zymo Clean and Concentrator kit, the ends digested with NgoM IV and BstE II (New England Biolabs), purified with the Zymo kit again and cloned upstream of the transposon in pTnLac as described below.

Plasmid pTnLac was digested with NgoM IV and BstE II to remove the ptac promoter and transposase and the fragments separated on an agarose gel. The band corresponding to the vector and transposon was excised, purified from the agarose, and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs) to prevent self-annealing. The enzyme was removed from the vector using a Zymo DNA Clean and Concentrator-5. The purified vector and CMVp/mATS/polyA were ligated together using a Stratagene T4 Ligase Kit and transformed into *E. coli* as described above.

Colonies resulting from this transformation were screened (mini-preps) as describe above and clones that were the correct size were verified by DNA sequence analysis as described above. The vector was given the name pTnMod (SEQ ID NO:3) and includes the following components:

Base pairs 1-130 are a remainder of F1(-) on from pBluescriptll sk(-) (Stratagene), corresponding to base pairs 1-130 of pBluescriptll sk(-).

Base pairs 131 - 132 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 133 -1777 are the CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems), corresponding to bp 229-1873 of pGWiz. The CMV promoter was modified by the addition of an ACC sequence upstream of ATG.

Base pairs 1778-1779 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 1780 - 2987 are the coding sequence for the transposase, modified from Tn10 (GenBank accession J01829) by optimizing codons for stability of the transposase mRNA and for the expression of protein. More specifically, in each of the codons for the first ten amino acids of the transposase, G or C was changed to A or T when such a substitution would not alter the amino acid that was encoded.

Base pairs 2988-2993 are two engineered stop codons.

Base pair 2994 is a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 2995 - 3410 are a synthetic polyA sequence taken from the pGWiz vector (Gene Therapy Systems), corresponding to bp 1922-2337 of 10 pGWiz.

Base pairs 3415 - 3718 are non-coding DNA that is residual from vector pNK2859.

Base pairs 3719 - 3761 are non-coding λ DNA that is residual from pNK2859.

Base pairs 3762 - 3831 are the 70 bp of the left insertion sequence recognized by the transposon Tn10.

Base pairs 3832-3837 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 3838 - 4527 are the multiple cloning site from pBluescriptll sk(20), corresponding to bp 924-235 of pBluescriptll sk(-). This multiple cloning site may be used to insert any coding sequence of interest into the vector.

Base pairs 4528-4532 are a residue from ligation of restriction enzyme sites used in constructing the vector.

Base pairs 4533 - 4602 are the 70 bp of the right insertion sequence recognized by the transposon Tn10.

Base pairs 4603 - 4644 are non-coding λ DNA that is residual from pNK2859.

Base pairs 4645 - 5488 are non-coding DNA that is residual from pNK2859.

Base pairs 5489 - 7689 are from the pBluescriptll sk(-) base vector - (Stratagene, Inc.), corresponding to bp 761-2961 of pBluescriptll sk(-).

Completing pTnMod is a pBlueScript backbone that contains a colE I origin of replication and an antibiotic resistance marker (ampicillin).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

All plasmid DNA was isolated by standard procedures. Briefly, *Escherichia coli* containing the plasmid was grown in 500 mL aliquots of LB broth (supplemented with an appropriate antibiotic) at 37°C overnight with shaking. Plasmid DNA was recovered from the bacteria using a Qiagen Maxi-Prep kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was resuspended in 500 μL of PCR-grade water and stored at -20°C until used.

EXAMPLE 2

Preparation of Transposon-Based Vector pTnMCS

Another transposon based vector was designed for inserting a desired coding sequence into the genome of eukaryotic cells. This vector was termed pTnMCS and its constituents are provided below.The sequence of the pTnMCS vector is provided in SEQ ID NO:2. The pTnMCS vector contains an avian optimized polyA sequence operably-linked to the transposase gene. The avian optimized polyA sequence contains approximately 40 nucleotides that precede the A nucleotide string.

Bp 1 – 130 Remainder of F1 (-) ori of pBluescriptII sk(-) (Stratagene) bp1-130

Bp 133 – 1777 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp 229-1873

Bp 1783 – 2991 Transposase, from Tn10 (GenBank accession #J01829) bp 108-1316

Bp 2992 – 3344 Non coding DNA from vector pNK2859

Bp 3345 – 3387 Lambda DNA from pNK2859

Bp 3388 – 3457 70 bp of IS10 left from Tn10

Bp 3464 – 3670 Multiple cloning site from pBluescriptII sk(-), thru the XmaI site bp 924-718

Bp 3671 - 3715 Multiple cloning site from pBluescriptII sk(-), from the XmaI site thru the XhoI site. These base pairs are usually lost when cloning into pTnMCS bp 717-673

Bp 3716 – 4153 Multiple cloning site from pBluescriptII sk(-), from the XhoI site bp 672-235

Bp 4159 - 4228 70 bp of IS10 right from Tn10

Bp 4229 - 4270 Lambda DNA from pNK2859

Bp 4271 – 5114 Non-coding DNA from pNK2859

Bp 5115 - 7315 pBluescript sk (-) base vector (Stratagene, Inc.) bp 761-2961.

EXAMPLE 3

Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/p146/PA) – Chicken

A vector is designed for inserting a p146 gene under the control of a chicken ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:38.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptll sk(-).

Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are an engineered stop codon.

Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922- 2337 of pGWiz.

Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are λ DNA that is residual from pNK2859.

Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptll sk(-).

Base pairs 4050 - 4951 are a chicken ovalbumin promoter (including SDRE) that corresponds to base pairs 431-1332 of the chicken ovalbumin promoter in GenBank Accession Number J00895 M24999.

Base pairs 4958 - 6115 are a chicken ovalbumin signal sequence and Ovalbumin gene that correspond to base pairs 66-1223 of GenBank Accession Number V00383.1 (The STOP codon being omitted).

Base pairs 6122 - 6271 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

Base pairs 6272 – 6316 are a p146 sequence (synthetic) with 2 added stop codons.

Base pairs 6324 – 6676 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272of pGWiz.

Base pairs 6682 - 7114 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptll sk(-).

Base pairs 7120- 7189 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7190 - 7231 are λ DNA that is residual from pNK2859.

Base pairs 7232 – 8096 are non coding DNA that is residual from pNK2859.

Base pairs 8097 - 10297 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961of pBluescriptll sk(-).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

EXAMPLE 4

Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/p146/PA) – Quail

A vector is designed for inserting a p146 gene under the control of a quail ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:39.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptll sk(-).

Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are an engineered stop codon.

Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922-2337 of pGWiz.

Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are λ DNA that is residual from pNK2859.

Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptll sk(-).

Base pairs 4050 - 4938 are the Japanese quail ovalbumin promoter (including SDRE, steroid-dependent response element). The Japanese quail ovalbumin promoter was isolated by its high degree of homology to the chicken ovalbumin promoter (GenBank accession number J00895 M24999, base pairs 431-1332).

Bp 4945 - 6092 are a quail ovalbumin signal sequence and ovalbumin gene that corresponds to base pairs 54 – 1201 of GenBank accession number X53964.1. (The STOP codon being omitted).

Base pairs 6097 - 6246 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

Base pairs 6247 – 6291 are a p146 sequence (synthetic) with 2 added stop codons.

Base pairs 6299 – 6651 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272of pGWiz.

Base pairs 6657 - 7089 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptll sk(-).

Base pairs 7095- 7164 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7165 - 7206 are λ DNA that is residual from pNK2859.

Base pairs 7207 – 8071 are non coding DNA that is residual from pNK2859.

Base pairs 8072 - 10272 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961of pBluescriptll sk(-).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

EXAMPLE 5

Preparation of Transposon-Based Vector pTnMod(Oval/ENT TAG/ProIns/PA) – Chicken

A vector was designed to insert a human proinsulin coding sequence under the control of a chicken ovalbumin promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:35.

Base pairs 1 - 130 are a remainder of F1(-) ori of pBluescriptII sk(-) (Stratagene) corresponding to base pairs 1-130 of pBluescriptll sk(-).

Base pairs 133 – 1777 are a CMV promoter/enhancer taken from vector pGWiz (Gene Therapy Systems) corresponding to base pairs 229-1873 of pGWiz.

Base pairs 1780 – 2987 are a transposase, modified from Tn10 (GenBank accession number J01829).

Base pairs 2988-2993 are two engineered stop codons.

Base pairs 2995 – 3410 are a synthetic polyA from pGWiz (Gene Therapy Systems) corresponding to base pairs 1922- 2337 of pGWiz.

Base pairs 3415 – 3718 are non coding DNA that is residual from vector pNK2859.

Base pairs 3719 – 3761 are λ DNA that is residual from pNK2859.

Base pairs 3762 – 3831 are the 70 base pairs of the left insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 3838 – 4044 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 924-718 of pBluescriptll sk(-).

Base pairs 4050 - 4951 are a chicken ovalbumin promoter (including SDRE) that corresponds to base pairs 431-1332 of the chicken ovalbumin promoter in GenBank Accession Number J00895 M24999.

Base pairs 4958 - 6115 are a chicken ovalbumin signal sequence and ovalbumin gene that correspond to base pairs 66-1223 of GenBank Accession Number V00383.1. (The STOP codon being omitted).

Base pairs 6122 - 6271 are a TAG sequence containing a gp41 hairpin loop from HIV I, an enterokinase cleavage site and a spacer (synthetic).

Base pairs 6272 – 6531 are a proinsulin gene.

Base pairs 6539 – 6891 are a synthetic polyadenylation sequence from pGWiz (Gene Therapy Systems) corresponding to base pairs 1920 - 2272of pGWiz.

Base pairs 6897 - 7329 are a multiple cloning site from pBlueScriptII sk(-) corresponding to base pairs 667-235 of pBluescriptll sk(-).

Base pairs 7335- 7404 are the 70 base pairs of the right insertion sequence (IS10) recognized by the transposon Tn10.

Base pairs 7405 - 7446 are λ DNA that is residual from pNK2859.

Base pairs 7447 – 8311 are non coding DNA that is residual from pNK2859.

Base pairs 8312 - 10512 are pBlueScript sk(-) base vector (Stratagene, Inc.) corresponding to base pairs 761-2961of pBluescriptll sk(-).

It should be noted that all non-coding DNA sequences described above can be replaced with any other non-coding DNA sequence(s). Missing nucleotide sequences in the above construct represent restriction site remnants.

EXAMPLE 6

Preparation of Transposon-Based Vector pTnMOD (CMV-CHOVg-ent-proinsulin-synPA)

A vector was designed to insert a proinsulin coding sequence under the control of a CMV promoter, and a ovalbumin gene including an ovalbumin signal sequence, into the genome of a bird given below as SEQ ID NO:36.

Bp 1 – 4045 from vector pTnMod, bp 1 - 4045

Bp 4051 – 5695 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1864

Bp 5702 –6855 Chicken ovalbumin gene taken from GenBank accession # V00383, bp 66-1219

Bp 6862 - 7011 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7012 – 7272 Human proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 7273 – 7317 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 7318 - 7670 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 7672 –11271 from cloning vector pTnMCS, bp 3716-7315

EXAMPLE 7

Transfection of Japanese Quail using a Transposon-based Vector containing a proinsulin Gene via Oviduct Injections

Two experiments were conducted in Japanese quail using transpson-based vectors containing either Oval promoter/Oval gene/GP41 Enterokinase TAG/proinsulin/Poly A (SEQ ID NO:35) or CMV promoter/Oval gene/GP41 Enterokinase TAG/proinsulin/Poly A (SEQ ID NO:36).

In the first experiment, the Oval promoter/Oval gene/GP41 Enterokinase TAG/proinsulin/Poly A containing construct was injected into the lumen of the oviduct of sexually mature quail; three hens received 5 μg at a 1:3 SUPERFECT® ratio and three received 10 μg at a 1:3 SUPERFECT® ratio. As of the writing of the present application, at least one bird that received above-mentioned construct was producing human proinsulin in egg white (other birds remain to be tested). This experiment indicates that 1) the DNA has been stable for at least 3 months; 2) protein levels are comparable to those observed with a constitutive promoter such as the CMV promoter; and 3) sexually mature birds can be injected and results obtained without the need for cell culture. It is estimated that each quail egg contains approximately 1.4 µg/ml of the proinsulin protein. It is also estimated that each transgenic chicken egg contains 50-75 mg of protein encoded by the gene of interest.

In the second experiment, the transposon-based vector containing CMV promoter/Oval gene/GP41 Enterokinase TAG/proinsulin/Poly A was injected into the lumen of the oviduct of sexually immature Japanese quail. A total of 9 birds were injected. Of the 8 survivors, 3 produced human proinsulin in the white of their eggs for over 6 weeks. An ELISA assay described in detail below was developed to detect GP41 in the fusion peptide (Oval gene/GP41 Enterokinase TAG/proinsulin) since the GP41 peptide sequence is unique and not found as part of normal egg white protein. In all ELISA assays, the same birds produced positive results and all controls worked as expected.

ELISA Procedure: Individual egg white samples were diluted in sodium carbonate buffer, pH 9.6, and added to individual wells of 96 well microtiter ELISA plates at a total volume of 0.1 ml. These plates were then allowed to coat overnight at 4°C. Prior to ELISA development, the plates were allowed warm to room temperature. Upon decanting the coating solutions and blotting away any excess, non-specific binding of antibodies was blocked by adding a solution of phosphate buffered saline (PBS), 1% (w/v) BSA, and 0.05% (v/v) Tween 20 and allowing it to incubate with shaking for a minimum of 45 minutes. This blocking solution was subsequently decanted and replaced with a solution of the primary antibody (Goat Anti-GP41 TAG) diluted in fresh PBS/BSA/Tween 20. After a two hour period of incubation with the primary antibody, each plate was washed with a solution of PBS and 0.05% Tween 20 in an automated plate washer to remove unbound antibody.

Next, the secondary antibody, Rabbit anti-Goat Alkaline Phosphatase-conjugated, was diluted in PBS/BSA/Tween 20 and allowed to incubate 1 hour. The plates were then subjected to a second wash with PBS/Tween 20. Antigen was detected using a solution of *p*-Nitrophenyl Phosphate in Diethanolamine Substrate Buffer for Alkaline Phosphatase and measuring the absorbance at 30 minutes and 1 hour.

EXAMPLE 8

Isolation of Human proinsulin Using Anti-TAG Column Chromotography

A HiTrap NHS-activated 1 mL column (Amersham) was charged with a 30 amino acid peptide that contained the gp-41 epitope containing gp-41's native disulfide bond that stabilizes the formation of the gp-41 hairpin loop. The 30 amino acid gp41 peptide is provided as SEQ ID NO:25. Approximately 10 mg of the peptide was dissolved in coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3 and the ligand was circulated on the column for 2 hours at room temperature at 0.5 mL/minute. Excess active groups were then deactivated using 6 column volumes of 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and the column was washed alternately with 6 column volumes of acetate buffer (0.1 M acetate, 0.5 M NaCl, pH 4.0) and ethanolamine (above). The column was neutralized using 1 X PBS. The column was then washed with buffers to be used in affinity purification: 75 mM Tris, pH 8.0 and elution buffer, 100 mM glycine-HCl, 0.5 M NaCl, pH 2.7. Finally, the column was equilibrated in 75 mM Tris buffer, pH 8.0.

Antibodies to gp-41 were raised in goats by inoculation with the gp-41 peptide described above. More specifically, goats were inoculated, given a booster injection of the gp-41 peptide and blood samples were obtained by veinupuncture. Serum was harvested by centrifugation. Approximately 30 mL of goat serum was filtered to 0.45 uM and passed over a TAG column at a rate of 0.5 mL/min. The column was washed with 75 mM Tris, pH 8.0 until absorbance at 280 nm reached a baseline. Three column volumes (3 mL) of elution buffer (100 mM glycine, 0.5 M NaCl, pH 2.7) was applied, followed by 75 mM Tris buffer, pH 8.0, all at a rate of 0.5 mL/min. One milliliter fractions were collected. Fractions were collected into 200 uL 1 M Tris, pH 9.0 to neutralize acidic factions as rapidly as possible. A large peak eluted from the column, coincident with the application the elution buffer. Fractions were pooled. Analysis by SDS-PAGE showed a high molecular weight species that separated into

two fragments under reducing condition, in keeping with the heavy and light chain structure of IgG.

Pooled antibody fractions were used to charge two 1 mL HiTrap NHS-activated columns, attached in series. Coupling was carried out in the same manner as that used for charging the TAG column.

Isolation of Ovalbumin-TAG-proinsulin from Egg White

Egg white from quail and chickens treated by intra-oviduct injection of the CMV-ovalbumin-TAG-proinsulin construct were pooled. Viscosity was lowered by subjecting the allantoid fluid to successively finer pore sizes using negative pressure filtration, finishing with a 0.22 μM pore size. Through the process, egg white was diluted approximately 1:16. The clarified sample was loaded on the Anti-TAG column and eluted in the same manner as described for the purification of the anti-TAG antibodies. A peak of absorbance at 280 nm, coincident with the application of the elution buffer, indicated that protein had been specifically eluted from the Anti-TAG column. Fractions containing the eluted peak were pooled for analysis.

The pooled fractions from the Anti-TAG affinity column were characterized by SDS-PAGE and western blot analysis. SDS-PAGE of the pooled fractions revealed a 60 kDal molecular weight band not present in control egg white fluid, consistent with the predicted molecular weight of the transgenic protein. Although some contaminating bands were observed, the 60 kDal species was greatly enriched compared to the other proteins. An aliquot of the pooled fractions was cleaved overnight at room temperature with the protease, enterokinase. SDS-PAGE analysis of the cleavage product, revealed a band not present in the uncut material that co-migrated with a commercial human proinsulin positive control. Western blot analysis showed specific binding to the 60 kDal species under non-reducing condition (which preserved the hairpin epitope of gp-41 by retaining the disulfide bond). Western analysis of the low molecular weight species that appeared upon cleavage with an anti-human proinsulin antibody, conclusively identified the cleaved fragment as human proinsulin.

EXAMPLE 9

Construction of a Transposon-based Transgene for the Expression of a Monoclonal Antibody

Production of a monoclonal antibody using transposon-based transgenic methodology is accomplished in a variety of ways.

1) two vectors are constructed: one that encodes the light chain and a second vector that encodes the heavy chain of the monoclonal antibody. These vectors are then incorporated into the genome of the target animal by at least one of two methods: a) direct transfection of a single animal with both vectors (simultaneously or as separate events); or, b) a male and a female of the species carry in their germline one of the vectors and then they are mated to produce progeny that inherit a copy of each.

2) the light and heavy chains are included on a single DNA construct, either separated by insulators and expression is governed by the same (or different) promoters, or by using a single promoter governing expression of both transgenes with the inclusion of elements that permit separate transcription of both transgenes, such as an internal ribosome entry site.

The following example describes the production of a transposon-based DNA construct that contains both the coding region for a monoclonal light chain and a heavy chain on a single construct. Beginning with the vector pTnMod, the coding sequences for the heavy and light chains are added, each preceded by an appropriate promoter and signal sequence. Using methods known to one skilled in the art, approximately 1 Kb of the proximal elements of the ovalbumin promoter are linked to the signal sequence of ovalbumin or some other protein secreted from the target tissue. Two copies of the promoter and signal sequence are added to the multiple cloning site of pTnMod, leaving space and key restriction sites between them to allow the subsequent addition of the coding sequences of the light and heavy chains of the monoclonal antibody. Methods known to one skilled in the art allow the coding sequences of the light and heavy chains to be inserted in-frame for appropriate expression. For example, the coding sequence of light and heavy chains of a murine monoclonal antibody that show specificity for human seminoprotein have recently been disclosed (GenBank Accession numbers AY129006 and AY129304 for the light and heavy chains, respectively). The light chain cDNA sequence is provided in SEQ

ID NO:54, whereas the cDNA of the heavy chain is reported as provided in SEQ ID NO:55.

Thus one skilled in the art can produce both the heavy and light chains of a monoclonal antibody in a single cell within a target tissue and species. If the modified cell contained normal posttranslational modification capabilities, the two chains would form their native configuration and disulfide attachments and be substrates for glycosylation. Upon secretion, then, the monoclonal antibody is accumulated, for example, in the egg white of a chicken egg, if the transgenes are expressed in the magnum of the oviduct.

It should also be noted that, although this example details production of a full-length murine monoclonal antibody, the method is quite capable of producing hybrid antibodies (e.g. a combination of human and murine sequences; 'humanized' monoclonal antibodies), as well as useful antibody fragments, known to one skilled in the art, such as Fab, Fc, F(ab) and Fv fragments. This method can be used to produce molecules containing the specific areas thought to be the antigen recognition sequences of antibodies (complementarity determining regions), linked, modified or incorporated into other proteins as desired.

EXAMPLE 10

Treatment of rats with a transposon-based vector for tissue-specific insulin gene incorporation

Rats are made diabetic by administering the drug streptozotocin (Zanosar; Upjohn, Kalamazoo, MI) at approximately 200 mg/kg. The rats are bred and maintained according to standard procedures. A transposon-based vector containing a proinsulin gene, an appropriate carrier, and, optionally, a transfection agent, are injected into rats' singhepatic (if using G6P) artery with the purpose of stable transformation. Incorporation of the insulin gene into the rat genome and levels of insulin expression are ascertained by a variety of methods known in the art. Blood and tissue samples from live or sacrificed animals are tested. A combination of PCR, Southern and Northern blots, *in-situ* hybridization and related nucleic acid analysis methods are used to determine incorporation of the vector-derived proinsulin DNA and levels of transcription of the corresponding mRNA in various organs and tissues of the rats. A combination of SDS-PAGE gels, Western Blot analysis, radioimmunoassay, and ELISA and other methods known to one of ordinary skill in

the art are used to determine the presence of insulin and the amount produced. Additional transfections of the vector are used to increase protein expression if the initial amounts of the expressed insulin are not satisfactory, or if the level of expression tapers off. The physiological condition of the rats is closely examined post-transfection to register positive or any negative effects of the gene therapy. Animals are examined over extended periods of time post-transfection in order to monitor the stability of gene incorporation and protein expression.

EXAMPLE 11

Optimization of Intra-oviduct and Intra-ovarian Arterial Injections

Overall transfection rates of oviduct cells in a flock of chicken or quail hens are enhanced by synchronizing the development of the oviduct and ovary within the flock. When the development of the oviducts and ovaries are uniform across a group of hens and when the stage of oviduct and ovarian development can be determined or predicted, timing of injections is optimized to transfect the greatest number of cells. Accordingly, oviduct development is synchronized as described below to ensure that a large and uniform proportion of oviduct secretory cells are transfected with the gene of interest.

Hens are treated with estradiol to stimulate oviduct maturation as described in Oka and Schimke (T. Oka and RT Schimke, J. Cell Biol., 41, 816 (1969)), Palmiter, Christensen and Schimke (J Biol. Chem. 245(4):833-845, 1970). Specifically, repeated daily injections of 1 mg estradiol benzoate are performed sometime before the onset of sexual maturation, a period ranging from 1 – 14 weeks of age. After a stimulation period sufficient to maximize development of the oviduct, hormone treatment is withdrawn thereby causing regression in oviduct secretory cell size but not cell number. At an optimum time after hormone withdrawal, the lumens of the oviducts of treated hens are injected with the transposon-based vector. Hens are subjected to additional estrogen stimulation after an optimized time during which the transposon-based vector is taken up into oviduct secretory cells. Re-stimulation by estrogen activates transposon expression, causing the integration of the gene of interest into the host genome. Estrogen stimulation is then withdrawn and hens continue normal sexual development. If a developmentally regulated promoter such as the ovalbumin promoter is used, expression of the transposon-based vector initiates

in the oviduct at the time of sexual maturation. Intra-ovarian artery injection during this window allows for high and uniform transfection efficiencies of ovarian follicles to produce germ-line transfections and possibly oviduct expression.

Other means are also used to synchronize the development, or regression, of the oviduct and ovary to allow high and uniform transfection efficiencies. Alterations of lighting and/or feed regimens, for example, cause hens to 'molt' during which time the oviduct and ovary regress. Molting is used to synchronize hens for transfection, and may be used in conjunction with other hormonal methods to control regression and/or development of the oviduct and ovary.

EXAMPLE 12

Additional Transposon-Based Vectors for Administration to an Animal

The following example provides a description of various transposon-based vectors of the present invention and several constructs for insertion into the transposon-based vectors of the present invention. These examples are not meant to be limiting in any way. The constructs for insertion into a transposon-based vector are provided in a cloning vector pTnMCS or pTnMod, both described above.

pTnMCS (CMV-CHOVg-ent-proinsulin-synPA) (SEQ ID NO:40)

Bp 1 – 3670 from vector PTnMCS, bp 1 - 3670

Bp 3676 – 5320 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems), bp 230-1864

Bp 5327 –6480 Chicken ovalbumin gene taken from GenBank accession # V00383, bp 66-1219

Bp 6487 - 6636 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6637 – 6897 Human proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 6898 – 6942 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 6943 - 7295 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 7296 – 10895 from cloning vector pTnMCS, bp 3716-7315.

pTnMOD (CMV-CHOVg-ent-proinsulin-synPA) (SEQ ID NO:36)

Bp 1 – 4045 from vector PTnMCS, bp 1 - 4045

Bp 4051 – 5695 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1864

Bp 5702 –6855 Chicken ovalbumin gene taken from GenBank accession # V00383, bp 66-1219

Bp 6862 - 7011 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7012 – 7272 Human proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 7273 – 7317 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 7318 - 7670 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 7672 –11271 from cloning vector pTnMCS, bp 3716-7315.

pTnMCS (CMV-prepro-ent-proinsulin-synPA)

Bp 1 – 3670 from vector PTnMCS, bp 1 - 3670

Bp 3676 – 5320 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems), bp 230-1864

Bp 5326 - 5496 Capsite/prepro taken fron GenBank accession # X07404, bp 563–733

Bp 5504 - 5652 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 5653 – 5913 Human proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 5914 – 5958 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 5959-6310 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 6313–9912 from cloning vector pTnMCS, bp 3716-7315.

pTnMCS(Chicken OVep+OVg'+ENT+proins+syn polyA)

Bp 1–3670 from vector pTnMCS, bp 1 - 3670

Bp 3676–4350 Chicken Ovalbumin enhancer taken from GenBank accession #S82527.1 bp 1-675

Bp 4357–5692 Chicken Ovalbumin promoter taken from GenBank accession # J00895M24999 bp 1-1336

Bp 5699–6917 Chicken Ovalbumin gene from GenBank Accession # V00383.1 bp 2-1220. (This sequence includes the 5'UTR, containing putative cap site, bp 5699-5762.)

Bp 6924-7073 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7074-7334 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7335-7379 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7380-7731 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7733–11332 from vector pTnMCS, bp 3716 – 7315.

<u>pTnMCS(Chicken OVep+prepro+ENT+proins+syn polyA)</u>

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

Bp 3676 – 4350 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675

Bp 4357 – 5692 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336

Bp 5699–5869 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

Bp 5876 - 6025 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6026 - 6286 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6287 - 6331 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6332 - 6683 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 6685 – 10284 from cloning vector pTnMCS, bp 3716 – 7315.

pTnMCS(Quail OVep+OVg'+ENT+proins+syn polyA)

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

Bp 3676 – 4333 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-house from quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession # S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative tochicken, so the number of bases does not correspond exactly.)

Bp 4340 – 5705 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

Bp 5712 – 6910 Quail Ovalbumin gene, EMBL accession # X53964, bp 1-1199. (This sequence includes the 5'UTR, containing putative cap site bp 5712-5764.)

Bp 6917 - 7066 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7067 - 7327 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7328 - 7372 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7373 - 7724 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7726 – 11325 from cloning vector pTnMCS, bp 3716 – 7315.

pTnMCS(Quail OVep+prepro+ENT+proins+syn polyA)

Bp 1 – 3670 from cloning vector pTnMCS, bp 1 - 3670

Bp 3676 – 4333 Quail Ovalbumin enhancer: 658 bp sequence, amplified from quail genomic DNA, roughly equivalent to the far- upstream chicken ovalbumin enhancer, GenBank accession #S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

Bp 4340 – 5705 Quail Ovalbumin promoter: 1366 bp sequence, amplified from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions

and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

Bp 5712–5882 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

Bp 5889 - 6038 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6039 - 6299 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6300 - 6344 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6345 - 6696 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 6698 – 10297 from cloning vector pTnMCS, bp 3716 - 7315.

pTnMOD (CMV-prepro-ent-proins-synPA)

Bp 1 – 4045 from vector PTnMCS, bp 1 - 4045

Bp 4051 – 5695 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1864

Bp 5701-5871 Capsite/prepro taken from GenBank accession # X07404, bp 563–733

Bp 5879 - 6027 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6028–6288 Human proinsulin taken from GenBank accession # NM000207, bp 117-377

Bp 6289 – 6333 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and pGWIZ (Gene Therapy Systems)

Bp 6334 - 6685 Synthetic polyA from the cloning vector pGWIZ (Gene Therapy Systems), bp 1920-2271

Bp 6687 –10286 from cloning vector pTnMCS, bp 3716-7315.

pTnMOD(Chicken OVep+OVg'+ENT+proins+syn polyA)

Bp 1 – 4045 from cloning vector pTnMod, bp 1 - 4045

Bp 4051 – 4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675

Bp 4732 – 6067 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336

Bp 6074 – 7292 Chicken Ovalbumin gene from GenBank Accession # V00383.1 bp 2-1220. (This sequence includes the 5'UTR, containing putative cap site bp 6074-6137.)

Bp 7299 - 7448 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7449 - 7709 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7710 - 7754 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7755 - 8106 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 8108 – 11707 from cloning vector pTnMod, bp 3716 – 7315.

<u>pTnMOD(Chicken OVep+prepro+ENT+proins+syn polyA)</u>

Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045

Bp 4051 – 4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1 bp 1-675

Bp 4732 – 6067 Chicken Ovalbumin promoter taken from GenBank accession # J00895-M24999 bp 1-1336

Bp 6074–6244 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

Bp 6251 - 6400 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6401 - 6661 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6662 - 6706 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6707 - 7058 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7060 – 10659 from cloning vector pTnMCS, bp 3716 – 7315.

<u>pTnMOD(Quail OVep+OVg'+ENT+proins+syn polyA)</u>

Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045

Bp 4051 – 4708 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-house from quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession # S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

Bp 4715 – 6080 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

Bp 6087 – 7285 Quail Ovalbumin gene, EMBL accession # X53964, bp 1-1199. (This sequence includes the 5'UTR, containing putative cap site bp 6087-6139.)

Bp 7292 - 7441 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 7442 - 7702 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 7703 - 7747 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 7748 - 8099 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 8101 – 11700 from cloning vector pTnMCS, bp 3716 – 7315.

pTnMOD(Quail OVep+prepro+ENT+proins+syn polyA)

Bp 1 – 4045 from cloning vector pTnMCS, bp 1 - 4045

Bp 4051 – 4708 Quail Ovalbumin enhancer: 658 bp sequence, amplified in-housefrom quail genomic DNA, roughly equivalent to the far-upstream chicken ovalbumin enhancer, GenBank accession #S82527.1, bp 1-675. (There are multiple base pair substitutions and deletions in the quail sequence, relative to chicken, so the number of bases does not correspond exactly.)

Bp 4715 – 6080 Quail Ovalbumin promoter: 1366 bp sequence, amplified in-house from quail genomic DNA, roughly corresponding to chicken ovalbumin promoter, GenBank accession # J00895-M24999 bp 1-1336. (There are multiple base pair substitutions and deletions between the quail and chicken sequences, so the number of bases does not correspond exactly.)

Bp 6087–6257 Cecropin cap site and prepro, Genbank accession # X07404 bp 563-733

Bp 6264 - 6413 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6414 - 6674 Human proinsulin GenBank Accession # NM000207 bp 117-377

Bp 6675 - 6719 Spacer DNA, derived as an artifact from the cloning vectors pTOPO Blunt II (Invitrogen) and gWIZ (Gene Therapy Systems)

Bp 6720 - 7071 Synthetic polyA from the cloning vector gWIZ (Gene Therapy Systems) bp 1920 - 2271

Bp 7073 – 10672 from cloning vector pTnMCS, bp 3716 – 7315.

<u>pTnMOD (CMV-prepro-ent-hGH-CPA)</u>

Bp 1–4045 from vector PTnMOD, bp 1 - 4045

Bp 4051–5694 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1873

Bp 5701-5871 Capsite/prepro taken fron GenBank accession # X07404, bp 563–733

Bp 5878-6012 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6013–6666 Human growth hormone taken from GenBank accession # V00519, bp 1-654

Bp 6673–7080 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 7082–10681 from cloning vector pTnMOD, bp 4091-7690.

<u>pTnMCS (CHOVep-prepro-ent-hGH-CPA)</u>

Bp 1–3670 from vector PTnMCS, bp 1-3670

Bp 3676–4350 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1, bp 1–675

Bp 4357-5692 Chicken Ovalbumin promoter taken from GenBank accession # J00899-M24999, bp 1-1336

Bp 5699–5869 Capsite/prepro taken fron GenBank accession # X07404, bp 563–733

Bp 5876-6010 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6011–6664 Human growth hormone taken from GenBank accession # V00519, bp 1-654

Bp 6671–7078 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 7080–10679 from cloning vector pTnMCS, bp 3716-7315.

pTnMCS (CMV-prepro-ent-hGH-CPA)

Bp 1 – 3670 from vector PTnMCS, bp 1 - 3670

Bp 3676–5319 CMV promoter/enhancer taken from vector pGWIZ (Gene therapy systems), bp 230-1873

Bp 5326-5496 Capsite/prepro taken fron GenBank accession # X07404, bp 563 – 733

Bp 5503-5637 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 5638–6291 Human growth hormone taken from GenBank accession # V00519, bp 1-654

Bp 6298–6705 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 6707–10306 from cloning vector pTnMCS, bp 3716-7315.

pTnMOD (CHOVep-prepro-ent-hGH-CPA)

Bp 1–4045 from vector PTnMOD, bp 1-4045

Bp 4051–4725 Chicken Ovalbumin enhancer taken from GenBank accession # S82527.1, bp 1–675

Bp 4732-6067 Chicken Ovalbumin promoter taken from GenBank accession # J00899-M24999, bp 1-1336

Bp 6074–6244 Capsite/prepro taken fron GenBank accession # X07404, bp 563–733

Bp 6251-6385 Synthetic spacer sequence and hairpin loop of HIV gp41 with an added enterokinase cleavage site

Bp 6386–7039 Human growth hormone taken from GenBank accession # V00519, bp 1-654

Bp 7046–7453 Conalbumin polyA taken from GenBank accession # Y00407, bp 10651-11058

Bp 7455–11054 from cloning vector pTnMOD, bp 4091-7690.

pTnMod(CMV/Transposase/ChickOvep/prepro/ProteinA/ConpolyA)

BP 1-130 remainder of F1 (-) ori of pBluescriptII sk(-) (Stragagene) bp 1-130.

BP 133-1777 CMV promoter/enhancer taken from vector pGWIZ (Gene Therapy Systems) bp 229-1873.

BP 1780-2987 Transposase, modified from Tn10 (GenBank #J01829).

BP 2988-2993 Engineered DOUBLE stop codon.

BP 2994-3343 non coding DNA from vector pNK2859.

BP 3344-3386 Lambda DNA from pNK2859.

BP 3387-3456 70bp of IS10 left from Tn10.

BP 3457-3674 multiple cloning site from pBluescriptII sk(-) bp 924-707.

BP 3675-5691 Chicken Ovalbumin enhancer plus promoter from a Topo Clone 10 maxi 040303 (5' XmaI, 3' BamHI)

BP 5698-5865 prepro with Cap site amplified from cecropin of pMON200 GenBank # X07404 (5'BamHI, 3'KpnI)

BP 5872-7338 Protein A gene from GenBank# J01786, mature peptide bp 292-1755 (5'KpnI, 3'SacII)

BP 7345-7752 ConPolyA from Chicken conalbumin polyA from GenBank # Y00407 bp 10651-11058. (5'SacII, 3'XhoI)

BP 7753-8195 multiple cloning site from pBluescriptII sk(-) bp 677-235.

BP 8196-8265 70 bp of IS10 left from Tn10.

BP 8266-8307 Lamda DNA from pNK2859

BP 8308-9151 noncoding DNA from pNK2859

BP 9152-11352 pBluescriptII sk(-) base vector (Stratagene, INC.) bp 761-2961 .

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.

Appendix A

SEQ ID NO:1 (modified Kozak sequence)
ACCATG

SEQ ID NO:2 (pTnMCS)
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121 ccacgttcgc cggcatcaga ttggctattg gccattgcat acgttgtatc catatcataa
181 tatgtacatt tatattggct catgtccaac attaccgcca tgttgacatt gattattgac
241 tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata tggagttccg
301 cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt
361 gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca
421 atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc
481 aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta
541 catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac
601 catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg
661 atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg
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781 acggtgggag gtctatataa gcagagctcg tttagtgaac cgtcagatcg cctggagacg
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ATTAATTGCG TTGCGCTCAC TGCCCGCTTT CCAGTCGGGA AACCTGTCGT 5700
GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG CGGTTTGCGT 5750
ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT 5800
TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT 5850
CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG 5900
CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG 5950
GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT 6000
GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC 6050
TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC 6100

CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA 6150
GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC 6200
GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT 6250
TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG 6300
GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG 6350
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CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG 6500
CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC 6550
TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG 6600
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TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG 6700
TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC 6750
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GAGGGCTTAC CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG 6850
CTCACCGGCT CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG 6900
AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT 6950
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CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA 7050
TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC 7100
CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT 7150
CAGAAGTAAG TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC 7200
ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT 7250
GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG 7300
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CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT 7550
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AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCAC 7689

SEQ ID NO:4 (conalbumin polyA)
tctgccattg ctgcttcctc tgcccttcct cgtcactctg aatgtggctt cttcgctact
gccacagcaa gaaataaaat ctcaacatct aaatgggttt cctgaggttt ttcaagagtc
gttaagcaca ttccttcccc agcacccctt gctgcaggcc agtgccaggc accaacttgg
ctactgctgc ccatgagaga aatccagttc aatattttcc aaagcaaaat ggattacata
tgccctagat cctgattaac aggcgtttgt attatctagt gctttcgctt cacccagatt
atcccattgc ctccc

SEQ ID NO:5 (synthetic polyA)
GGCGCCTGGATCCAGATCACTTCTGGCTAATAAAAGATCAGAGCTCTAGAGATCTGTGTGTTGGTTTTT
TGTGGATCTGCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACC
CTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGG
TGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGCACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGG
CATGCTGGGGATGCGGTGGGCTCTATGGGTACCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
TCTCGGTACCTCTCTC

SEQ ID NO:6 (avian optimized polyA)
ggggatcgc tctagagcga tccgggatct cgggaaaagc gttggtgacc aaaggtgcct
tttatcatca ctttaaaaat aaaaaacaat tactcagtgc ctgttataag cagcaattaa
ttatgattga tgcctacatc acaacaaaaa ctgatttaac aaatggttgg tctgccttag
aaagtatatt tgaacattat cttgattata ttattgataa taataaaaac cttatcccta
tccaagaagt gatgcctatc attggttgga atgaacttga aaaaaattag ccttgaatac
attactggta aggtaaacgc cattgtcagc aaattgatcc aagagaacca a

SEQ ID NO:7
(vitellogenin promoter)

TGAATGTGTT CTTGTGTTAT CAATATAAAT CACAGTTAGT GATGAAGTTG GCTGCAAGCC
TGCATCAGTT CAGCTACTTG GCTGCATTTT GTATTTGGTT CTGTAGGAAA TGCAAAAGGT
TCTAGGCTGA CCTGCACTTC TATCCCTCTT GCCTTACTGC TGAGAATCTC TGCAGGTTTT
AATTGTTCAC ATTTTGCTCC CATTTACTTT GGAAGATAAA ATATTTACAG AATGCTTATG
AAACCTTTGT TCATTTAAAA ATATTCCTGG TCAGCGTGAC CGGAGCTGAA AGAACACATT
GATCCCGTGA TTTCAATAAA TACATATGTT CCATATATTG TTTCTCAGTA GCCTCTTAAA
TCATGTGCGT TGGTGCACAT ATGAATACAT GAATAGCAAA GGTTTATCTG GATTACGCTC
TGGCCTGCAG GAATGGCCAT AAACCAAAGC TGAGGGAAGA GGGAGAGTAT AGTCAATGTA
GATTATACTG ATTGCTGATT GGGTTATTAT CAGCTAGATA ACAACTTGGG TCAGGTGCCA
GGTCAACATA ACCTGGGCAA AACCAGTCTC ATCTGTGGCA GGACCATGTA CCAGCAGCCA
GCCGTGACCC AATCTAGGAA AGCAAGTAGC ACATCAATTT TAAATTTATT GTAAATGCCG
TAGTAGAAGT GTTTTACTGT GATACATTGA AACTTCTGGT CAATCAGAAA AAGGTTTTTT
ATCAGAGATG CCAAGGTATT ATTTGATTTT CTTTATTCGC CGTGAAGAGA ATTTATGATT
GCAAAAAGAG GAGTGTTTAC ATAAACTGAT AAAAAACTTG AGGAATTCAG CAGAAAACAG
CCACGTGTTC CTGAACATTC TTCCATAAAA GTCTCACCAT GCCTGGCAGA GCCCTATTCA
CCTTCGCT

SEQ ID NO:8 (fragment of ovalbumin promoter - chicken)
GAGGTCAGAAT GGTTTCTTTA CTGTTTGTCA ATTCTATTAT TTCAATACAG
AACAATAGCT TCTATAACTG AAATATATTT GCTATTGTAT ATTATGATTG
TCCCTCGAAC CATGAACACT CCTCCAGCTG AATTTCACAA TTCCTCTGTC
ATCTGCCAGG CCATTAAGTT ATTCATGGAA GATCTTTGAG GAACACTGCA
AGTTCATATC ATAAACACAT TTGAAATTGA GTATTGTTTT GCATTGTATG
GAGCTATGTT TTGCTGTATC CTCAGAAAAA AAGTTTGTTA TAAAGCATTC
ACACCCATAA AAAGATAGAT TTAAATATTC CAGCTATAGG AAAGAAAGTG
CGTCTGCTCT TCACTCTAGT CTCAGTTGGC TCCTTCACAT GCATGCTTCT
TTATTTCTCC TATTTTGTCA AGAAAATAAT AGGTCACGTC TTGTTCTCAC
TTATGTCCTG CCTAGCATGG CTCAGATGCA CGTTGTAGAT ACAAGAAGGA
TCAAATGAAA CAGACTTCTG GTCTGTTACT ACAACCATAG TAATAAGCAC
ACTAACTAAT AATTGCTAAT TATGTTTTCC ATCTCTAAGG TTCCCACATT
TTTCTGTTTT CTTAAAGATC CCATTATCTG GTTGTAACTG AAGCTCAATG
GAACATGAGC AATATTTCCC AGTCTTCTCT CCCATCCAAC AGTCCTGATG
GATTAGCAGA ACAGGCAGAA AACACATTGT TACCCAGAAT TAAAAACTAA
TATTTGCTCT CCATTCAATC CAAAATGGAC CTATTGAAAC TAAAATCTAA
CCCAATCCCA TTAAATGATT TCTATGGCGT CAAAGGTCAA ACTTCTGAAG
GGAACCTGTG GGTGGGTCAC AATTCAGGCT ATATATTCCC CAGGGCTCAG

SEQ ID NO:9 (chicken ovalbumin ehancer)
ccgggctgca gaaaaatgcc aggtggacta tgaactcaca tccaaaggag cttgacctga
tacctgattt tcttcaaact ggggaaacaa cacaatccca caaaacagct cagagagaaa
ccatcactga tggctacagc accaaggtat gcaatggcaa tccattcgac attcatctgt
gacctgagca aaatgattta tctctccatg aatggttgct tctttccctc atgaaaaggc
aatttccaca ctcacaatat gcaacaaaga caaacagaga acaattaatg tgctccttcc
taatgtcaaa attgtagtgg caaagaggag aacaaaatct caagttctga gtaggtttta
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aaagtgcttt tataactttc aggtctccga gtctttattc atgagactgt tggtttaggg
acagacccac aatgaaatgc ctggcatagg aaagggcagc agagccttag ctgacctttt
cttgggacaa gcattgtcaa acaatgtgtg acaaaactat ttgtactgct ttgcacagct
gtgctgggca gggcaatcca ttgccaccta tcccaggtaa ccttccaact gcaagaagat
tgttgcttac tctctctaga

SEQ ID NO:10 (5' untranslated region)
GTGGATCAACATACAGCTAGAAAGCTGTATTGCCTTTAGCACTCAAGCTCAAAAGACAACTCAGAGTTC
ACC

SEQ ID NO:11 (putative cap site)
ACATACAGCTAG AAAGCTGTAT TGCCTTTAGC ACTCAAGCTC AAAAGACAAC TCAGAGTTCA

SEQ ID NO:12 (Chicken Ovalbumin Signal Sequence)
ATG GGCTCCATCG GCGCAGCAAG CATGGAATTT TGTTTTGATG TATTCAAGGA GCTCAAAGTC
CACCATGCCA ATGAGAACAT CTTCTACTGC CCCATTGCCA TCATGTCAGC TCTAGCCATG
GTATACCTGG GTGCAAAAGA CAGCACCAGG ACACAGATAA ATAAGGTTGT TCGCTTTGAT
AAACTTCCAG GATTCGGAGA CAGTATTGAA GCTCAGTGTG GCACATCTGT AAACGTTCAC
TCTTCACTTA GAGACATCCT CAACCAAATC ACCAAACCAA ATGATGTTTA TTCGTTCAGC
CTTGCCAGTA GACTTTATGC TGAAGAGAGA TACCCAATCC TGCCAGAATA CTTGCAGTGT
GTGAAGGAAC TGTATAGAGG AGGCTTGGAA CCTATCAACT TTCAAACAGC TGCAGATCAA
GCCAGAGAGC TCATCAATTC CTGGGTAGAA AGTCAGACAA ATGGAATTAT CAGAAATGTC
CTTCAGCCAA GCTCCGTGGA TTCTCAAACT GCAATGGTTC TGGTTAATGC CATTGTCTTC
AAAGGACTGT GGGAGAAAAC ATTTAAGGAT GAAGACACAC AAGCAATGCC TTTCAGAGTG
ACTGAGCAAG AAAGCAAACC TGTGCAGATG ATGTACCAGA TTGGTTTATT TAGAGTGGCA
TCAATGGCTT CTGAGAAAAT GAAGATCCTG GAGCTTCCAT TTGCCAGTGG GACAATGAGC
ATGTTGGTGC TGTTGCCTGA TGAAGTCTCA GGCCTTGAGC AGCTTGAGAG TATAATCAAC
TTTGAAAAAC TGACTGAATG GACCAGTTCT AATGTTATGG AAGAGAGGAA GATCAAAGTG
TACTTACCTC GCATGAAGAT GGAGGAAAAA TACAACCTCA CATCTGTCTT AATGGCTATG
GGCATTACTG ACGTGTTTAG CTCTTCAGCC AATCTGTCTG GCATCTCCTC AGCAGAGAGC
CTGAAGATAT CTCAAGCTGT CCATGCAGCA CATGCAGAAA TCAATGAAGC AGGCAGAGAG
GTGGTAGGGT CAGCAGAGGC TGGAGTGGAT GCTGCAAGCG TCTCTGAAGA ATTTAGGGCT
GACCATCCAT TCCTCTTCTG TATCAAGCAC ATCGCAACCA ACGCCGTTCT CTTCTTTGGC
AGATGTGTTT CCCCT

SEQ ID NO:13 (Chicken Ovalbumin Signal Sequence - shortened 50bp)
ATG GGCTCCATCG GCGCAGCAAG CATGGAATTT TGTTTTGATG TATTCAAGGA

SEQ ID NO:14 (Chicken Ovalbumin Signal Sequence - shortened 100bp)
ATG GGCTCCATCG GCGCAGCAAG CATGGAATTT TGTTTTGATG TATTCAAGGA GCTCAAAGTC
CACCATGCCA ATGAGAACAT CTTCTACTGC CCCATTGCCA

SEQ ID NO:15 (vitellogenin targeting sequence)
ATGAGGGGGATCATACTGGCATTAGTGCTCACCCTTGTAGGCAGCCAGAAGTTTGACATTGGT

SEQ ID NO:16 (pro-insulin sequence)
TTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGC
TTCTTCTACACACCCAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGG
GGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAA
CAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTCTGCAACTAG

SEQ ID NO:17 (p146 protein)
KYKKALKKLAKLL

SEQ ID NO:18 (p146 coding sequence)
AAATACAAAAAAGCACTGAAAAAACTGGCAAAACTGCTG

SEQ ID NO:19 (spacer)
$(GPGG)_x$

SEQ ID NO:20 (spacer)
GPGGGPGGGPGG

SEQ ID NO:21 (spacer)
GGGGSGGGGSGGGGS

SEQ ID NO:22 (spacer)
GGGGSGGGGSGGGGSGGGGS

SEQ ID NO:23 (repeat domain in TAG spacer sequence)
Pro Ala Asp Asp Ala

SEQ ID NO:24 (TAG spacer sequence)
Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp

SEQ ID NO:25 (gp41 epitope)
Ala Thr Thr Cys Ile Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu

SEQ ID NO:26 (polynucleotide sequence encoding gp41 epitope)

Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Thr Thr Cys Ile Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu Asp Asp Asp Asp Lys

SEQ ID NO:27 (enterokinase cleavage site)
DDDDK

SEQ ID NO:28 (TAG sequence)
Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Pro Ala Asp Asp Ala Thr Thr Cys Ile Leu Lys Gly Ser Cys Gly Trp Ile Gly Leu Leu Asp Asp Asp Asp Lys

SEQ ID NO:29 (altered transposase Hef forward primer)
ATCTCGAGACCATGTGTGAACTTGATATTTTACATGATTCTCTTTACC

SEQ ID NO:30 (altered transposase Her reverse primer)
GATTGATCATTATCATAATTTCCCCAAAGCGTAACC

SEQ ID NO:31 (Xho I restriction site)
CTCGAG

SEQ ID NO:32 (Bcl I restriction site)
TGATCA

SEQ ID NO:33 (CMVf-NgoM IV primer)
TTGCCGGCATCAGATTGGCTAT

SEQ ID NO:34 (Syn-polyAr-BstE II primer)
AGAGGTCACCGGGTCAATTCTTCAGCACCTGGTA

SEQ ID NO:35 (pTnMod(Oval/ENT tag/Proins/PA) - Chicken)

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CTGACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG   50
CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC  100
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CATGTCCAAC ATTACCGCCA TGTTGACATT GATTATTGAC TAGTTATTAA  250
TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG  300
CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC  350
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CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCCC CCTATTGACG  500
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10081 cggaagggcc gagcgcagaa gtggtcctgc aactttatcc gcctccatcc agtctattaa
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10801 catgagcgga tacatatttg aatgtattta gaaaaataaa caaataggggg ttccgcgcac
10861 atttccccga aaagtgccac

SEQ ID NO:41 (cecropin prepro)
AAT TTC TCA AGG ATA TTT
TTC TTC GTG TTC GCT TTG
GTT CTG GCT TTG TCA ACA
GTT TCG GCT GCG CCA GAG
CCG AAA

SEQ ID NO:42 (cecropin prepro extended)
AAT TTC TCA AGG ATA TTT
TTC TTC GTG TTC GCT TTG
GTT CTG GCT TTG TCA ACA
GTT TCG GCT GCG CCA GAG
CCG AAA TGG AAA GTC TTC
AAG

SEQ ID NO:43 (cecropin pro)
GCG CCA GAG CCG AAA

SEQ ID NO:44 (cecropin pro extended)
GCG CCA GAG CCG AAA TGG AAA GTC TTC AAG

SEQ ID NO:45 (a Kozak sequence)
ACCATGG

SEQ ID NO:46 (a Kozak sequence)
ACCATGT

SEQ ID NO:47 (a Kozak sequence)
AAGATGT

SEQ ID NO:48 (a Kozak sequence)
ACGATGA

SEQ ID NO:49 (a Kozak sequence)
AAGATGG

SEQ ID NO:50 (a Kozak sequence)
GACATGA

SEQ ID NO:51 (a Kozak sequence)

ACCATGA

SEQ ID NO:52 (a Kozak sequence)
ACCATGT

SEQ ID NO:53 (Vit pro/Vit targ/TAG/pro-insulin/synthetic polyA)
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TGCATCAGTT CAGCTACTTG GCTGCATTTT GTATTTGGTT CTGTAGGAAA TGCAAAAGGT
TCTAGGCTGA CCTGCACTTC TATCCCTCTT GCCTTACTGC TGAGAATCTC TGCAGGTTTT
AATTGTTCAC ATTTTGCTCC CATTTACTTT GGAAGATAAA ATATTTACAG AATGCTTATG
AAACCTTTGT TCATTTAAAA ATATTCCTGG TCAGCGTGAC CGGAGCTGAA AGAACACATT
GATCCCGTGA TTTCAATAAA TACATATGTT CCATATATTG TTTCTCAGTA GCCTCTTAAA
TCATGTGCGT TGGTGCACAT ATGAATACAT GAATAGCAAA GGTTTATCTG GATTACGCTC
TGGCCTGCAG GAATGGCCAT AAACCAAAGC TGAGGGAAGA GGGAGAGTAT AGTCAATGTA
GATTATACTG ATTGCTGATT GGGTTATTAT CAGCTAGATA ACAACTTGGG TCAGGTGCCA
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GCCGTGACCC AATCTAGGAA AGCAAGTAGC ACATCAATTT TAAATTTATT GTAAATGCCG
TAGTAGAAGT GTTTTACTGT GATACATTGA AACTTCTGGT CAATCAGAAA AAGGTTTTTT
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CCTTCGCTAT GAGGGGGATC ATACTGGCAT TAGTGCTCAC CCTTGTAGGC AGCCAGAAGT
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GCCCTGGTGC AGGCAGCCTG CAGCCCTTGG CCCTGGAGGGG TCCCTGCAGA AGCGTGGCAT
TGTGGAACAA TGCTGTACCA GCATCTGCTC CCTCTACCAGC TGGAGAACTA CTGCAACTAG
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TGTGGATCTGCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACC
CTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGG
TGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGCACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGG
CATGCTGGGGATGCGGTGGGCTCTATGGGTACCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
TCTCGGTACCTCTCTC

SEQ ID NO:54 (exemplary antibody light chain sequence)
1 gagctcgtga tgacccagac tccatcctcc ctgtctgcct ctctgggaga cagagtcacc
61 atcagttgca gggcaaatca ggacattagc aattatttaa actggtatca gcagaaacca
121 gatggaactg ttaaactcct gatctactac acatcaagat tacactcagg ggtcccatca
181 aggttcagtg gcagtgggtc tggaacagat tattctctca ccattagcaa cctggagcaa
241 gaagattttg ccacttactt ttgccaacag ggtaatacgc ttccgtggac gttcggtgga
301 ggcaccaacc tggaaatcaa acgggctgat gctgcaccaa ctgtatccat cttcccacca
361 tccagtgagc agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
421 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa tggcgtcctg
481 aacagttgga ctgatcagga cagcaaagac agcacctaca gcatgagcag caccctcacg
541 ttgaccaagg acgagtatga acgacataac agctatacct gtgaggccac tcacaagaca
601 tcaacttcac ccattgtcaa gagcttcaac aggaatgagt gttaa

SEQ ID NO:55 (exemplary antibody heavy chain sequence)
1 ctcgagtcag gacctggcct ggtggcgccc tcacagaacc tgtccatcac ttgcactgtc
61 tctgggtttt cattaaccag ctatggtgta cactgggttc gccagcctcc aggaaagggt
121 ctggaatggc tgggagtaat atggactggt agaagcacaa cttataattc ggctctcatg

181 tccagactga gcatcagcaa agacaactcc aagagccaag ttttcttaaa aatgaacagt
241 ctgcaaactg atgacacagc catttactac tgtggcagag gggtctgat tacgtccttt
301 gctatggact actggggtca aggaacctca gtcaccgtct cctcagccaa aacgacaccc
361 ccatctgtct atccactggc ccctggatct gctgcccaaa ctaactccat ggtgaccctg
421 ggatgcctgg tcaagggcta tttccctgag ccagtgacag tgacctggaa ctctggatcc
481 ctgtccagcg gtgtgcacac cttcccagct gtcctgcagt ctgacctcta cactctgagc
541 agctcagtga ctgtcccctc cagcacctgg cccagcgaga ccgtcacctg caacgttgcc
601 cacccggcca gcagcaccaa ggtggacaag aaaattgtgc ccagggattg tactagt

GENE REGULATION IN TRANSGENIC ANIMALS USING A TRANSPOSON-BASED VECTOR

ABSTRACT

Administration of modified transposon-based vectors has been used to achieve stable incorporation of exogenous genes into animals. These transgenic animals produce transgenic progeny. Further, these transgenic animals produce large quantities of desired molecules encoded by the transgene. Transgenic egg-laying animals produce large quantities of desired molecules encoded by the transgene and deposit these molecules in the egg.

Attorney Docket No. 51687-0260P (294995)

FIGURE 1

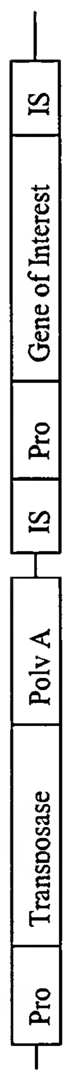
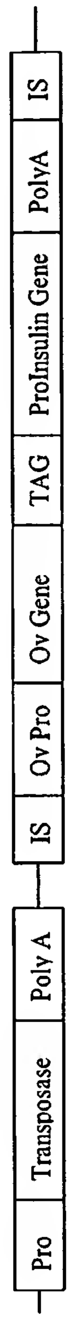


FIGURE 2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Transposase
Poly A
IS
Ov Pro
Ov Gene
TAG
ProInsulin Gene
PolyA
IS

FIGURE 3

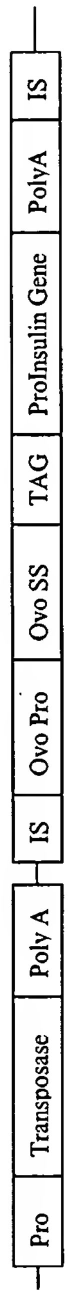

Pro
Transposase
Poly A
IS
Ovo Pro
Ovo SS
TAG
ProInsulin Gene
PolyA
IS

FIGURE 4


Vit Pro
Transposase
Poly A
IS
Vit Pro
Vit Targ
ProInsulin
Poly A
IS

FIGURE 5

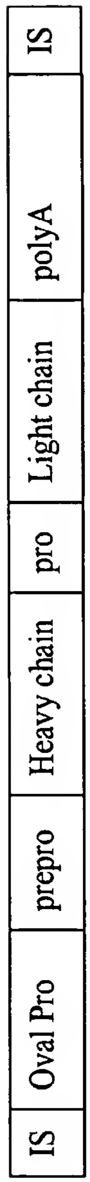


FIGURE 6

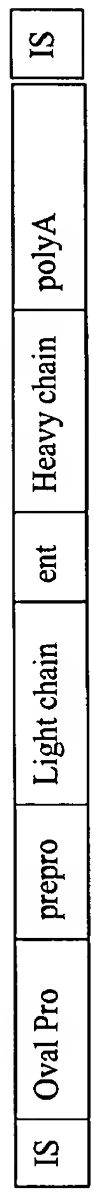
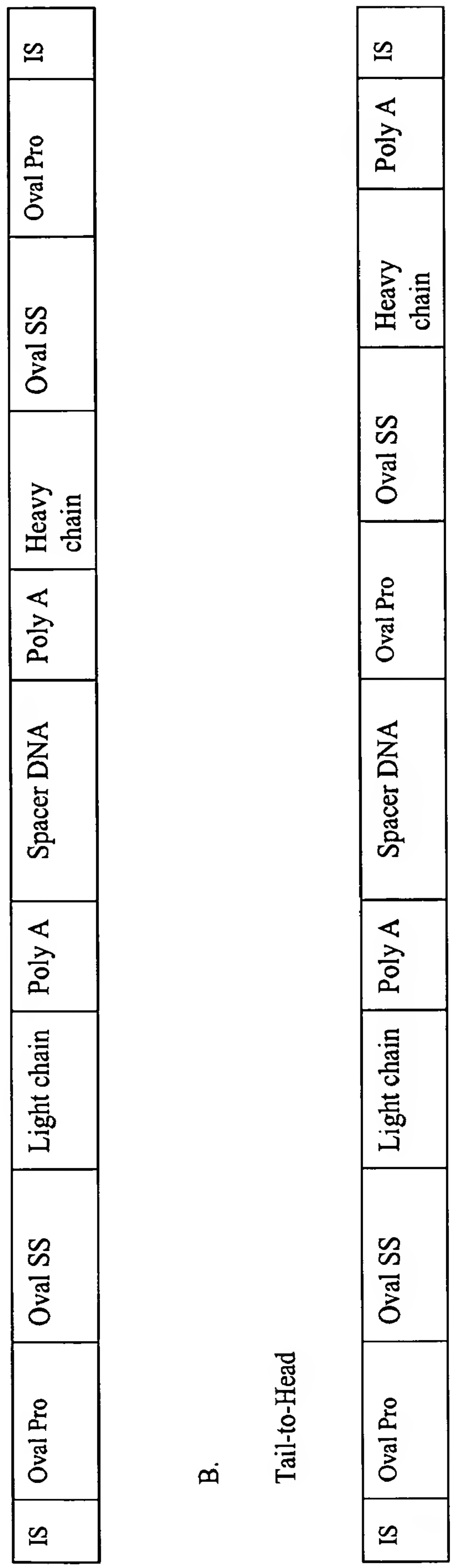


FIGURE 7

A. Tail-to-Tail

B. Tail-to-Head



BEST AVAILABLE COPY

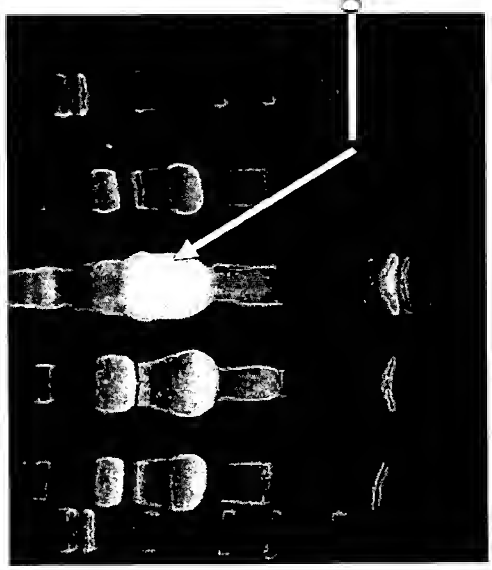


FIGURE 8

FIGURE 9